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(54) Title: ANTIBODIES AGAINST HUMAN CD40

(57) Abstract

The Applicants have discovered novel chimeric and humanized anti–human CD40 antibodies which block the interaction between gp39 and CD40. The anti–CD40 antibodies of the present invention are effective in modulating humoral immune responses against T cell–dependent antigens, collagen induced arthritis, and skin transplantation, and are also useful for their anti–inflammatory properties.

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ANTIBODIES AGAINST HUMAN CD40

Background of the Invention

Immune/inflammatory responses are mediated by a complex series of interactions. One receptor/ligand pair shown to be important in these processes is CD40/gp39. The gp39/CD40 interaction mediates a number of important signaling events between activated T cells and other effector cells of the immune system leading to amplification of an immune/inflammatory response. Responses to signaling through CD40 include T cell help to B cells in the humoral immune response, induction of cytokines by monocytes, and expression of adhesion molecules by endothelial cells.

CD40 is a type I cell surface receptor and a member of the tumor necrosis factor receptor (TNFR) supergene family. Though originally identified as a B cell antigen, CD40 is now believed to be expressed by all antigen presenting cells (APC), including dendritic cells, keratinocytes, and monocytes. CD40 is also expressed by cell types that can act as APC under certain conditions, such as vascular endothelial cells, or cells involved in direct interactions with T cells or T cell precursors such as thymic epithelial cells. More recently, it has also been reported that CD40 can be expressed by fibroblasts, eosinophils, and activated T cells. CD40 expression has also been seen in cancerous cells. Evidence for this is primarily derived from the identification of some carcinoma and melanoma derived cell lines which are $CD40^+$. (Clark and Ledbetter, Proc. Natl. Acad. Sci. (1986) 83:4494-98; Schriever et al., J. Exp. Med. (1989) 169:2043-58; Caux et al., J. Exp. Med. (1994) 180:1263-72; Alderson et al., J. Exp. Med. (1993) 178:669-74; Young et al., Int. J. Cancer (1989) 43:786-94; Paulie et al., Cancer Immunol. Immunother. (1985) 20:23-28; Denfeld et al., Eur. J. Immunol. (1996) 26:2329-34; Gaspari et al., Eur. J. Immunol. (1996) 26:1371-77; Peguet-Navarro et al., J. Immunol. (1997) 158:144-52; Hollenbaugh et al., J. Exp. Med. (1995) 182:33-40; Galy and Spits, J. Immunol. (1992) 149:775-82;

T cells. Gp39 is also known as CD40L, TRAP, T-BAM, and now has the official CD designation from the Leukocyte Workshop of CD154. In *in vitro* assays, gp39 appears on the T cells approximately 2-4 hours following T cell activation and levels peak at 6-8 hours. The protein level then rapidly declines and is undetectable 24 hours after stimulation. Gp39 expression has also been detected on eosinophils and mast cells. (Noelle et al., Proc. Natl. Acad. Sci. (1992) 89:6550-54; Hollenbaugh et al., EMBO J. (1992) 11:4313-21; Spriggs et al., J. Exp. Med. (1992) 176:1543-50; Graf et al., Eur. J. Immunol. (1992) 22:3191-94; Covey et al., Mol. Immunol. (1994) 31:471-84; Castle et al., J. Immunol. (1993) 151:1777-88; Roy et al., J. Immunol. (1993) 151:2497-2510; Gauchat et al., Nature (1993) 365:340-43; Gauchat et al., Eur. J. Immunol. (1995) 25:863-65; Koshy et al., J. Clin. Invest. (1996) 98:826-37; Desai-Mehta et al., J. Clin. Invest. (1996) 97:2063-73).

CD40 is a potent signaling receptor, providing a mechanism for activated T-cells to regulate a wide range of immune and inflammatory responses. *In vitro* and *in vivo* studies with recombinant forms of the gp39 ligand and with anti-CD40 mAbs have shown that signaling through this receptor leads to a cellular response in all known $CD40^+$ cells, and that outcome not only varies by cell type but is also modulated by concurrent signaling events through other receptors. In B cells, for example, CD40 signaling in conjunction with signaling by the IL-4 receptor leads to B cell proliferation and production of antibodies of the IgE isotype, while CD40 signaling in conjunction with signals from the IL-10 receptor lead to B cell proliferation and production of antibodies of the IgG isotype (Gordon et al., Eur. J. Immunol. (1987) 17:1535-38; Rousset et al., J. Exp. Med. (1991) 173:705-710; Jabara et al., J. Exp. Med. (1990) 172:1861-64; Gascan et al., J. Immunol. (1991) 147:8-13). Gp39 mediated CD40 signaling may play a role in cellular immunity through the induction of CD80 and CD86, important T cell costimulatory molecules which bind CD28 and CTLA4 (Goldstein et al., Mol. Immunol. (1996) 33:541-52).

The CD40/gp39 receptor/ligand system is one of the many systems which are involved in the productive interaction between activated T cells and other cells of the immune system. However, a number of findings suggest that this interaction is unique and central to the regulation of the humoral immune response in humans. In

particular, defects in gp39 expression or structure have been shown to be the cause of the human immunodeficiency known as X-linked hyper IgM (X-HIM) syndrome. This immunodeficiency is characterized by the inability of affected individuals to produce antibodies other than those of the IgM isotype, indicating that the productive interaction between gp39 and CD40 is required for an effective humoral immune response (Allen et al., Science (1993) 259:990-93; Aruffo et al., Cell (1993) 72:291-300; Di Santo et al., Nature (1993) 361:541-43; Fuleihan, et al., Proc. Natl. Acad. Sci. (1993) 90(6):2170-73; Korthauer et al., Nature (1993) 361:539-541; Notarangelo et al., Immunodef. Rev. (1992) 3:101-22). Likewise, recent data indicate that non-X-linked HIM syndrome in humans is caused by defects in the CD40 molecule. Using gene knockout technology, mice lacking CD40 or gp39 have been generated. These mice exhibit a phenotype which has the same characteristics as HIM syndrome suggesting that mice can be an appropriate model in which to test the effects of *in vivo* treatment with either anti-CD40 or anti-gp39 mAbs that block the interaction between CD40 and gp39 (Kawabe et al., Immunity (1994) 1:167-78; Xu et al., Immunity (1994) 1:423-431; Renshaw et al., J. Exp. Med. (1994) 180:1889-1900; Castigli et al., Proc. Natl. Acad. Sci. USA (1994) 91:12135-39).

The effects of *in vivo* inhibition of the CD40/gp39 interaction have been extensively studied in normal mice and mouse models of disease using a hamster anti-mouse gp39 mAb (MR1). The immunosuppressive capacity of the antibody is reflected in its ability to completely inhibit the humoral immune response to T-cell dependent antigens (Foy, et al., J. Exp. Med. (1993) 178:1567-75). Several mouse models of immune diseases have also been shown to be inhibited by treatment with the antibody, including those mediated by cellular immune responses. Disease models shown to be inhibited by treatment with anti-gp39 include collagen induced arthritis, experimental allergic encephalomyelitis, lupus nephritis, transplant rejection, and graft vs. host disease (Durie et al., Science (1993) 261:1328-30; Berry, et al., unpublished; Gerritse et al., Proc. Natl. Acad. Sci. USA (1995) 93:2499-504; Mohan et al., J. Immunol. (1995) 154:1470-1480; Larsen et al., Transplantation (1996) 61:4-9; Hancock et al., Proc. Natl. Acad. Sci. USA (1996) 93:13967-72; Parker et al., Proc. Natl. Acad. Sci. USA (1995) 92:9560-64; Durie, et al., J. Clin. Invest. (1994)

94:1333-38; Wallace, et al., unpublished). The role of CD40/gp39 in the amplification of a cellular immune response may be direct, through the stimulation of a subset of activated T cells that are capable of expressing CD40, or indirect, through induction of cytokines and the expression of important co-stimulatory cell surface molecules such as CD80 and CD86, which bind to the T cell receptors CD28 and CTLA-4. The anti-inflammatory effects of the inhibitor have been demonstrated by studies in a mouse model of oxygen-induced lung injury. The effects on inflammation *in vivo* are suggested by the *in vitro* results demonstrating stimulation of CD40 on vascular endothelial cells and monocytes which results in the expression of cell adhesion molecules, nitric oxide (NO), matrix metalloproteinases and proinflammatory cytokines (Kiener et al., J. Immunol. (1995) 155:4917-25; Malik et al., J. Immunol. (1995) 156:3952-60; Hollenbaugh et al., J. Exp. Med. (1995) 182:33-40).

Studies with anti-human gp39 mAbs in monkeys have shown that biologics which inhibit the interaction between gp39 and CD40 *in vivo* are effective immunosuppressive agents in primates. Anti-gp39 mAbs have been demonstrated to be effective in the inhibition of antibody responses to T-cell dependent antigens, and to protect allografts from rejection, results analogous to that seen in rodents.

Collectively the above studies have shown that agents which disrupt the interaction between gp39 and CD40 would be potent immunosuppressive and anti-inflammatory agents. Therefore, there exists a need in the art for an effective method of blocking the CD40/gp39 interaction to provide an immunosuppressive or anti-inflammatory effect. A purpose of the present invention is to provide an antibody which blocks the interaction between gp39 and CD40.

Another object of the present invention is to provide a chimeric antibody effective in blocking the interaction between CD40 and gp39.

An additional object of the present invention is to provide a humanized antibody effective in blocking the interaction between CD40 and gp39.

A further object of the present invention is a method of modulating an immune response by administering an antibody, chimeric antibody, or humanized antibody of

the present invention. The method may be useful in treating any number of autoimmune diseases, as well as skin or other organ transplantation.

Summary of the Invention

The present invention comprises a novel antibody, more preferably a chimerized anti-human CD40 monoclonal antibody (mAb), which blocks the interaction between gp39 and CD40. In one embodiment of the present invention, a particularly preferred chimerized anti-human CD40 mAb is referred to as "chi220". Chi220 is a chimeric antibody comprising murine variable and human kappa and gamma 1 constant regions. Chi220, like its parent mouse mAb, binds to CD40 and, as a result, effectively blocks humoral immune responses to T cell-dependent antigens in a dose dependent fashion.

Also encompassed within the scope of the present invention are humanized anti-CD40 antibodies which block the interaction between gp39 and CD40. In one embodiment of the present invention, a humanized antibody is referred to as F4; in another embodiment the humanized antibody is referred to as L3.17. The preferred humanized antibodies of the present invention comprise human variable heavy and variable light regions with murine CDR's grafted therein.

The anti-CD40 antibodies of the present invention, preferably the chimeric and humanized antibodies disclosed herein, are effective in modulating humoral immune responses against T cell-dependent antigens, collagen induced arthritis, and transplant rejection. The anti-CD40 antibodies of the present invention, preferably the chimeric and humanized antibodies disclosed herein, are also useful for their anti-inflammatory properties (which are similar to those seen with anti-gp39).

The antibodies of the present invention, particularly the anti-CD40 chimeric antibody chi220 and the anti-CD40 humanized antibodies F4 and L3.17, have wide therapeutic applications, including autoimmune diseases, inflammatory diseases and transplantation. Because of the expression of CD40 seen on malignant cells of several histologic types, the potential oncology applications of anti-CD40 antibodies, particularly the chimeric and humanized antibodies of the present invention, are evident.

The following abbreviations are used throughout the present application and are known by those skilled in the art: APC (antigen presenting cell); CDR (complemenrarity- determining region); CHO (chinese hamster ovary); CIA (collagen-induced arthritis); Cmax (maximum serum concentration); COS (African green monkey fibroblast cell line); DMARD (disease modifying anti-rheumatic drugs); ELISA (enzyme-linked immunosorbent assay); EPT (end point titers); EU (endotoxin units); Fab (antigen binding fragment); FITC (fluoroisothiocyanate); Hu (humanized); h106-2 (humanized anti-gp39 mAb); HAMA (human-anti-mouse antibodies); im (intramuscular); KLH (keyhole limpet hemocyanin); mAb (monoclonal antibody); MTX (methotrexate); OVA (ovalbumin); PBS (phosphate buffered saline); PCR (polymerase chain reaction); PE (phycoerytherin); sc (subcutaneous); SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis); SEC (size exclusion chromatography); SRBC (sheep red blood cells); STR (stirred tank reactor); TNF (tumor necrosis factor); VL (antibody light chain variable region); VH (antibody heavy chain variable region).

A nucleic acid encoding a preferred light chain of a chimeric antibody of the present invention (chimeric antibody 2.220) has been deposited with the American Type Culture Collection and given the Accession Number ATCC _____. A nucleic acid encoding a preferred heavy chain of a chimeric antibody of the present invention (2.220) has been deposited with the American Type Culture Collection and given the Accession Number ATCC _____.

A nucleic acid encoding a preferred light chain of a humanized antibody of the present invention (humanized antibody F4) has been deposited with the American Type Culture Collection and given the Accession Number ATCC _____. A nucleic acid encoding an additional preferred light chain of a humanized antibody of the present invention (humanized antibody L3.17) has been deposited with the American Type Culture Collection and given the Accession Number ATCC _____. A nucleic acid encoding a preferred heavy chain of a humanized antibody of the present invention (F4 and L3.17) has been deposited with the American Type Culture Collection and given the Accession Number ATCC _____.

The deposit(s) referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-Organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence(s) of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

All references cited in this application, whether *supra* or *infra*, are herein incorporated by reference in their entirety.

Brief Description of the Drawings

Figure 1 shows the inhibition of sgp39 binding to Raji cells by anti-human CD40 mAbs.

Figure 2 is a schematic outlining the primate study protocol. Days of treatment are indicated with diamonds. Immunizations with SRBC and KLH are indicated with rectangles and triangles, respectively. Animals treated with 2.36 were not studied past Phase I and animals treated with 1.106 were not studied past Phase II.

Figure 3 shows the anti-SRBC antibody response in primates. Figure 3a shows the results of analysis for IgM anti-SRBC antibodies. Figure 3b shows the results of analysis for IgG anti-SRBC antibodies.

Figure 4a shows the sequence of the light chain variable region of chi220 in bold (SEQ ID NO:1), and Figure 4b shows the sequence of the heavy chain variable region of chi220 in bold (SEQ ID NO:2). The underlined sequences in Figure 4a and 4b are the inserted signal sequences of the human antibody with the closest homology which had been used as humanization template.

Figure 5 shows the results of *in vitro* assays testing chimeric and humanized antibody of the present invention. Figure 5a shows the binding of chi220 and h220v3 to hCD40-mG2b in an ELISA based assay. Figure 5b shows the inhibition of sgp39-mediated costimulation of human B cells with anti-human CD40 mAbs.

Figure 6 shows the IgM Anti-SRBC antibody response. Figure 6a shows the results from monkeys that received 10, 40 or 100 mg/kg chi220. Figure 6b shows the results from monkeys that received 0.1 or 1 mg/kg chi220.

Figure 7 shows the IgG Anti-SRBC antibody response. Figure 7a shows the results from monkeys that received 10, 40 or 100 mg/kg chi220. Figure 7b shows the results from monkeys that received 0.1 or 1 mg/kg chi220.

Figure 8 shows the anti-OVA antibody response in primates. Figure 8a shows the results of analysis for IgM anti-OVA antibodies. Figure 8b shows the results of analysis for IgG anti-OVA antibodies.

Figure 9 shows the anti-KLH antibody response in primates. Figure 9a shows the results of analysis for IgM anti-KLH antibodies. Figure 9b shows the results of analysis for IgG anti-KLH antibodies.

Figure 10 shows a comparison of the ability of antibody 7E1-G1 and 7E1-G2b to suppress an IgG antibody response to SRBC.

Figure 11 shows the dose response of inhibition of antibody response to SRBC with 7E1-G2b.

Figure 12 shows expression vector maps for a heavy chain region and light chain region of a chimeric antibody of the present invention.

Figure 13 provides a nucleic acid sequence for an expression vector capable of expressing a heavy chain of a chimeric antibody of the present invention. The start ATG (nucleotides 1000-1002), encoding the start Met of the inserted signal sequence of the human antibody, is in bold. Nucleotides 1057 through 1422 (SEQ ID NO:5), underlined, provide a preferred nucleic acid sequence encoding a variable heavy chain of an antibody of the present invention.

Figure 14 provides a nucleic acid sequence for an expression vector capable of expressing a light chain of a chimeric antibody of the present invention. The start ATG (nucleotides 1005-1007), encoding the start Met of the inserted signal sequence of the human antibody, is in bold. Nucleotides 1065 through 1388 (SEQ ID NO:6), underlined, provide a preferred nucleic acid sequence encoding a variable light chain of an antibody of the present invention.

Figure 15 shows an alignment of murine anti-CD40 variable regions and a human template sequences. The amino acid sequences of the murine anti-CD40 H and L chain variable regions were used to identify homologous human germline sequences. The numbering of residues and the definition of CDRs (underlined) were based on Kabat et al. (Kabat, E.A., et al., (1991) Sequences of proteins of immunological interest (5th Ed). Washington DC: United States Department of Health and Human Services; Kabat, E.A., et al., (1977) *J. Biol. Chem.* 252:6609-6616). Differences in sequence are indicated by vertical lines and framework positions characterized in the combinatorial expression library are marked with an asterisk.

Figure 16 shows the results of titration of humanized anti-CD40 variants on immobilized antigen. Bacterially-expressed chimeric anti-CD40 Fab and select variants from each of the libraries were characterized. Chimeric (filled circles), Hu I-19C11 (open circles), Hu II-CW43 (open squares), Hu III-2B8 (filled triangles), and an irrelevant (filled squares) Fab were released from the periplasmic space of 15 ml bacterial cultures and serial dilutions were incubated with CD40-Ig antigen immobilized on microtiter plates. Antibody binding was quantitated as described below.

Figure 17 demonstrates how antibody affinity correlates with the inhibition of soluble-gp39 binding to CD40-Ig. The ligand for the CD40 receptor, gp39, was captured in a microtiter plate. Subsequently, varying amounts of purified chimeric (filled circles), Hu II-CW43 (open squares), Hu III-2B8 (filled triangles), Hu II/III-2B12 (open circles), and irrelevant (filled squares) Fab were co-incubated with 2 μg/ml CD40-Ig on the microtiter plate. Binding of CD40-Ig to gp39 was quantitated as described below.

Figure 18 shows the quantitation of murine framework residues in active variants. The variable regions of the most active anti-CD40 variants from the framework optimization library Hu I (A) and from the framework/HCDR3 optimization library Hu II (B) were sequenced to identify the amino acids at framework library positions. Each unique variant was categorized based on the total number of murine residues retained at the 8 framework library positions. Thirty-four

clones from the Hu I library and fourteen clones from the Hu II library were sequenced, leading to the identification of 24 and 10 unique variants, respectively. The solid line indicates the sequence distribution expected from an equal number of randomly selected variants.

Detailed Description of the Invention

The present inventors have developed chimeric and humanized anti-human CD40 antibodies with immunosuppressive properties. Such anti-human CD40 antibodies have obvious applications as a therapeutic. The present inventors have also developed a closely matched anti-mouse CD40 mAb (closely matched to the anti-human CD40 mAb) which is useful to study the effects of anti-CD40 mAb therapy in a number of mouse models of immune and inflammatory disease. Development of anti-CD40 antibodies is complicated by the fact that CD40 is a potent signaling molecule. Antibodies that bind to this antigen can be categorized based on the ability to stimulate CD40 signaling as well as the ability to block the CD40/gp39 interaction.

Applicants' anti-human CD40 mAb, which blocks the CD40/gp39 interaction, was selected from an extensive panel of anti-CD40 mAbs. The antibody, labeled 2.220, was chimerized and humanized. "Chimeric" antibodies comprise a light chain and a heavy chain: the light chain is comprised of a light chain variable region and a light chain constant region; the heavy chain is comprised of a heavy chain variable region and a heavy chain constant region. Chimeric antibodies comprise variable regions from one species and constant regions from another species (for example, mouse variable regions joined to human constant regions). (See, e.g., U.S. Patents 4,816,397 and 4,816,567). Each of the light chain variable region (VL) and heavy chain variable region (VH) consists of "framework" regions interrupted by three hypervariable regions called "complementarity determining regions" or "CDRs". "Humanized" antibodies comprise antibodies with human framework regions combined with CDRs from a donor mouse or rat immunoglobulin. (See, e.g., U.S. Patent 5,530,101). Encompassed within the scope of the present invention are humanized antibodies which comprise CDRs derived from the murine variable chains disclosed herein.

The most straightforward approach to humanizing an antibody consists of grafting the CDRs from the donor mAb onto a human framework (Jones, P.T., et al., (1986) *Nature* 321:522-525). However, certain framework residues support CDR structure, and contact antigen grafting murine CDRs onto human framework templates may diminish the binding activity of the resulting humanized mAb (Foote, J., et al., (1992) *J. Mol. Biol.* 224:487-499). Assessing the potential contribution of specific framework residues to antibody affinity poses two problems. First, for a particular mAb it is difficult to predict which framework residues serve a critical role in maintaining the affinity and specificity. Second, for framework positions that differ between the parent mAb and the human template it is difficult to predict whether the amino acid derived from the murine parent or the human template will yield a more active mAb. Consequently, antibody humanization methods that rely exclusively on structural predictions are not always successful.

The prior art contains a description of a general antibody engineering strategy that addresses the difficulty of maintaining antibody binding activity following humanization (Rosok, M. J., et al., (1996) *J. Biol. Chem.* 271:22611-22618). Potentially important framework residues that differ between the parent mAb and the human template are characterized in a single step by synthesizing and expressing a combinatorial antibody library that contains all possible combinations of parent and human template amino acids at the framework positions in question. Variants displaying the optimal framework structure are identified by screening and subsequently, optimal framework structure(s) are determined by DNA sequencing. Typically, sequencing multiple active clones reveals critical framework positions that require the expression of a particular amino acid. Conversely, the expression of a murine or human amino acid at a library framework position at an equivalent frequency in the active clones is consistent with a less important function for that particular framework position. Thus, a humanized version of the antibody that preserves the binding activity of the parent mAb is rapidly identified based on functional binding.

The processes of antibody humanization and affinity maturation are often performed in discreet steps (Rosok (1996), *supra*; Yelton, D. E., et al., (1995) *J.*

Immunol. 155:1994-2004; Wu, H., et al., (1998) *Proc. Natl. Acad. Sci. USA* 95:6037-6042; Baca, M., et al., (1997) *J. Biol. Chem.* 272:10678-10684; Marks. J.D., et al., (1992) *J. Biol. Chem.* 267:16007-16010). Using a modified strategy described below, multiple humanized versions of the murine mAb 2.220 displaying affinities equivalent to or better than the chimeric Fab were generated.

Applicants' chimeric anti-CD40 antibody of the present invention is referred to herein as "chi220". Applicants' closely matched anti-mouse CD40 mAb is referred to herein as "7E1". Applicants' humanized anti-CD40 antibodies of the present invention are referred to herein as "F4" and "L3.17".

Two different isotype variants of 7E1 were generated. These two variants of 7E1 are useful in examining the role of the Fc portion of the molecule in anti-CD40 mAb therapy in preclinical models of immune and inflammatory diseases. The generation of the anti-mouse CD40 mAb, the criteria used to select one which matched the properties of chi220, the generation of the isotype variants of the mAb and their *in vivo* activity in mouse models of immune disease are also presented herein. Studies with both chi220 and its parent murine mAb 2.220 in monkeys, as well as studies with 7E1 in mice, showed that these anti-CD40 mAbs are potent immunosuppressive agents, and will be discussed in more detail below. The studies described herein were performed using standard technology known by those skilled in the art.

In summary, Applicants' antibodies have been shown to suppress a humoral immune response in monkeys. Likewise, two isotype variants of a closely matched anti-mouse CD40 mAb, 7E1, showed immunosuppressive activity in a number of preclinical models of human disease. Taken together, these findings indicate that chi220, F4 and L3.17 are useful for clinical application in the treatment of autoimmune diseases and transplantation.

The following examples are for illustrative purposes only and do not limit the scope of Applicants invention, which is defined only by the claims.

Example 1

Selection of Murine Anti-Human CD40 Antibody

A. Isolation and *In Vitro* Characterization

A panel of monoclonal antibodies was generated against human CD40 using standard hybridoma technology with human CD40 fusion protein as the immunogen. Antibodies were screened for binding to CD40 using both a $CD40^+$ cell line and fusion proteins. Assays of gp39 binding to CD40 and functional assays of stimulation through CD40 were used to characterize cloned antibodies. Selected antibodies were then characterized for crossreactivity with primate cells to assess the suitability of the antibodies for use in primate preclinical models.

1. Immunization and Fusion

Two fusions were performed to generate hybridomas producing anti-human CD40 mAbs. Immunizations to generate immune lymphocytes were carried out in 6-8 week old female BALB/c mice using as the immunogen a recombinant fusion protein consisting of the extracellular domain of human CD40 fused to the hinge, CH2 and CH3 domains of a murine IgG2b antibody (hCD40-mG2b).

For fusion 40-1, the mouse was initially immunized subcutaneously at 3-4 sites with an emulsion (total of 200 ul) of 30 ug hCD40-mG2b in complete Freund's adjuvant. The animal was similarly boosted on day 21 with hCD40-mG2b in incomplete Freund's adjuvant and then given a final pre-fusion immunization on day 37 by intravenous injection of 30 ug of hCD40-mG2b in PBS. Immunizations for fusion 40-2 were similarly performed except that Ribi adjuvant (R-730) was substituted for Freund's adjuvant. Booster immunizations were on days 21 and 42 with the final pre-fusion boost on day 58.

Three days following final booster injections, leukocytes from the spleen and lymph nodes were harvested and fused at a 3:1 ratio with X63-Ag8.653 mouse myeloma cells using standard methods (Kearney et al., J. Immunol. (1979) 123:1548-50; Lane, J. Immunol. (1985) 81:223-28). Cell suspensions from each fusion were seeded into ten 96-well cell culture plates at a plating density of approximately 170,000 total cells (pre-fusion) per well.

2. Screening and Cloning

Two assay formats were used to identify mAbs with specificity for native human CD40. Cell culture supernatants from all wells were initially screened for their ability to bind to a CD40 positive, EBV-transformed human B cell line (1A2-2C) in an ELISA-based format. Each supernatant was then tested in an ELISA based format for reactivity with a purified, recombinant fusion protein consisting of the extracellular domain of human CD40 fused to the hinge, CH2 and CH3 domains of a human IgG1 antibody, hCD40-Ig, and a similarly constructed irrelevant human Ig fusion protein, Leu8-hIg (Hollenbaugh, et al., EMBO J. (1992) 11:4313-4321). Reactivity with the former and not the latter fusion protein, coupled with the cell binding data, established the presence of antibody specific for native CD40 in approximately 200 master wells.

A key functional property for the desired anti-CD40 mAb was the capacity to completely block the interaction of CD40 and its ligand, gp39. Thus, as the next step in antibody selection, all CD40 specific master well supernatants were assessed for their ability to inhibit the binding of the soluble, recombinant murine CD8-human gp39 fusion protein, sgp39, to immobilized hCD40-Ig in an ELISA-based format. Those that completely inhibited this interaction were subsequently titrated in the same format to establish which wells contained the highest titer of inhibiting antibody. From this analysis, ten of the most strongly inhibiting master wells were selected for cloning.

Cloning of the appropriate antibody secreting cells was accomplished in a two step process. Cells from each master well were first "minicloned" at a seeding density of 10 cells/well after which the highest titered, CD40-specific "miniclone" well was formally cloned by a limiting dilution method.

3. Further Characterization

Six assay formats were used to further characterize the antibodies. These were inhibition of sgp39 binding to human B cells, inhibition of B cell proliferation induced by sgp39 plus anti-IgM, inhibition of *in vitro* antibody synthesis by B cells induced by activated T cells, direct costimulation of B cells with anti-IgM, costimulation of B cells with anti-IgM in the presence of cross-linking anti-kappa

light chain antibody, and costimulation of B cells with anti-IgM in the presence of a second anti-CD40 mAb, G28-5. This mAb was known to possess strong costimulatory activity and to incompletely block CD40/gp39 interaction. It has been included for comparison purposes in many of these assays.

This analysis led to the selection of four mAbs: 1.66 (IgG2b), 2.36 (IgG2a), 2.174 (IgG1) and 2.220 (IgG2a). Tests were run to characterize the mAbs. In one experiment, cells from the human B cell line Raji were incubated with 2 or 20 μg/ml of various anti-CD40 mAbs followed by a second incubation in undiluted COS cell supernatant containing mCD8-gp39 fusion protein (sgp39). Bound sgp39 was detected by further incubation of the cells with a FITC labeled anti-mCD8 mAb and analysis of the cells on a FACScan. Percent inhibition was calculated by dividing mean fluorescence of samples incubated with antibody by the mean fluorescence of samples without antibody in the first incubation (Figure 1).

As shown in Figure 1, each of these four mAbs was capable of completely inhibiting the binding of sgp39 fusion protein to a human B cell line expressing high levels of CD40, although in the case of 2.174, a relatively high concentration of antibody was required for complete blockade. Similar data were obtained using human tonsillar B cells. These data were paralleled by two functional assays. First, it was shown that each mAb was able to completely block sgp39-mediated costimulation of human tonsillar B cells. Second, each significantly inhibited the production of IgG and IgM in an *in vitro* T cell-dependent B cell antibody synthesis assay.

Three of the four antibodies showed limited ability to costimulate B cell proliferation in the presence of anti-IgM. MAb 2.220 was more consistent in its ability to induce weak costimulatory activity. With the addition of an anti-kappa light chain antibody, used to cross-link the anti-CD40 mAbs, 2.36 gained significant costimulatory activity, while the activity of other three antibodies was not affected. The costimulatory ability of G28-5 was shown to be differentially modulated when it was paired in combination with each of the four new anti-CD40 mAbs. MAbs 1.66 and especially 2.174 enhanced G28-5 costimulation, whereas 2.220 and 2.36 suppressed it.

Following selection based on evaluations in human *in vitro* systems, the four anti-CD40 mAbs were further examined for their suitability for *in vivo* evaluation in non-human primate studies. Two key points of analysis were the relative potency of each for binding to primate B cells and suppression of *in vitro*, T cell-dependent B cell antibody synthesis. It was found that all four mAbs crossreacted with cynomolgus macaque (*Macaca fascicularis*) B cells. 2.36 and 2.220 bound with higher avidity than 2.174 and 1.66. Lower apparent binding of mAbs 2.174 and 1.66 was not due to their particular isotypes, as other isotype-matched anti-CD40 mAbs demonstrated binding levels comparable to 2.36 and 2.220 (e.g., G28-5 and 2.118). These results were in contrast to that observed with human B cells where each of the mAbs demonstrated comparable binding . The ability of the four mAbs to suppress antibody synthesis by monkey B cells was found to parallel the ability to bind.

B. *In Vivo* Characterization

Two studies were performed in non-human primates using the murine anti-human CD40 mAbs to assess the suitability of anti-CD40 as an immunosuppressive agent and to select the appropriate antibody for further development. First, the *in vivo* clearance and acute toxicity of the four selected anti-CD40 mAbs were compared. These results were used to select two antibodies, 2.36 and 2.220, that were then tested in a second study designed to assess efficacy in the inhibition of the antibody response to a T-dependent antigen and acute toxicity.

Primate Efficacy Study with 2.36 and 2.220

Based upon previous findings, mAbs 2.36 and 2.220 were evaluated for their ability to suppress a T-dependent antibody response following intravenous administration to cynomolgus monkeys. This study was divided into three phases (Figure 2). In Phase I, four groups consisting of one or two male and two female cynomolgus monkeys each were immunized intravenously on day 1 with sheep red blood cells (SRBCs), and then treated with 20 mg/kg of mAb 2.36, 2.220, 1.106 (IgG1 murine anti-human gp39, positive control), or L6 (IgG2a murine anti-human tumor antigen, negative control) on days 1, 3, and 5. IgM and IgG titers to the SRBC immunogen, serum levels of test and control articles, the presence of anti-test and

control article antibodies, serum immunoglobulin levels, peripheral blood leukocyte counts, and the frequencies of various subpopulations of peripheral blood lymphocytes were determined. In phase II, after the control and test articles had cleared, the animals were immunized with SRBCs and a second antigen, keyhole limpet hemocyanin (KLH), to assess the induction of immunological tolerance and the reversibility of the observed immunosuppression. In phase III, selected animals were reimmunized to determine if the initially suppressed anti-SRBC antibody response recovered following an additional challenge with SRBCs and to assess the secondary antibody response to KLH.

An experiment was performed to show that MAb 2.220 significantly suppressed the primary antibody response to SRBCs (Figure 3). Monkeys were treated with 20 mg/kg of either mAb 1.106, L6, 2.36 or 2.220 on Phase I Days 1, 3, and 5. Monkeys were immunized with SRBC on Day 1 of Phase I, II and III. Figure 3a shows the results of serum samples that were analyzed for IgM anti-SRBC antibodies; Figure 3b shows the results of serum samples that were analyzed for IgG anti-SRBC antibodies. Data are expressed as the geometric mean anti-SRBC titer for each group (n=3 or 4).

The peak primary response was inhibited 85% and 98% for IgM and IgG, respectively. Following clearance of mAb 2.220 in serum to below detectable levels, the peak secondary response to SRBCs was still inhibited 79% and 56% for IgM and IgG, respectively, compared to the negative control response in Phase I. This was in contrast to the positive control, mAb 1.106, with which a strong secondary antibody response to SRBCs was observed. The tertiary response to SRBCs was not inhibited, indicating that mAb 2.220 induced a prolonged immunosuppression, but not immunological tolerance. All animals immunized with KLH had a primary and secondary anti-KLH response, suggesting that the immunosuppression was reversible. Animals treated with 2.36 were not included in phase II because there was no significant inhibition seen in phase I of the study.

Mean peak serum concentrations, occurring immediately after the last dose, were 744 and 405 μg/ml for mAbs 2.220 and 2.36, respectively. Whereas mAb 2.36

cleared from the serum to below detectable levels by day 15, mAb 2.220 did not clear until day 29. Both mAbs 2.36 and 2.220 were immunogenic.

There were no drug-related clinical observations, changes in body weight or food consumption, or alterations in hematology or serum Ig levels in any animal. The only drug-related findings observed were transient 70% and 43% decreases in the percentages of peripheral B cells with mAbs 2.36 and 2.220, respectively. Recovery of B cells to normal levels occurred within 2-3 weeks post-treatment.

In summary, mAb 2.220 significantly suppressed the antibody response to SRBCs and 2.36 did not. Although mAb 2.220 induced a prolonged antigen-specific immunosuppression, it was reversible. Based on these findings, mAb 2.220 was selected for further development.

Example 2

Generation of Chimeric Antibody chi220

To address immunogenicity of the murine anti-human mAb 2.220, recombinant forms in which variable regions are fused to human constant regions were generated and compared for in vitro efficacy. The two approaches used were generation of a chimeric antibody, containing the unaltered murine variable regions, and humanized forms in which murine hypervariable regions (CDRs) are grafted on human framework sequences within the variable regions. Chimeric antibodies retain the antigen binding properties of parent antibody, but may have a greater likelihood of being immunogenic. Humanized antibodies are less likely to be immunogenic, but mutations introduced in the humanization can affect antigen binding.

A. Construction and *In Vitro* Characterization of Chimeric and Humanized Antibodies

The VL and VH regions from the anti-CD40 mAb 2.220 were obtained by PCR. cDNA was generated from RNA isolated from the hybridoma expressing the 2.220 mAb using an IgG1-specific or a Cκ-specific anti-sense primer to obtain the VH or VL regions, respectively. A poly-G tail was added to these single stranded cDNAs. The variable regions were then amplified by PCR using as a sense primer an oligonucleotide containing a poly-C sequence, complimentary to the poly-G tail, and a

nested set of antisense primers. The PCR product obtained was then inserted into a bacterial vector using restriction sites included in the primers. Multiple clones were then sequenced by dideoxynucleotide sequencing. Two independent experiments were performed, beginning at the RNA stage and the sequences obtained were the same.

To generate a chimeric form of the antibody, the variable regions were amplified by PCR using primers that introduced a sequence encoding the signal sequence of the human antibody found to most closely match the 2.220 sequence, as shown in Figure 4. The underlined portions of the light chain variable sequence (Figure 4a) and the heavy chain variable sequence (Figure 4b) designate the inserted signal sequences of the human antibody with the closest homology to murine 2.220. These PCR products were inserted into a vector containing sequences encoding the constant regions of human kappa or of human γ1 to generate complete light or heavy chain, respectively. The vectors also contained appropriate drug resistance genes for the generation and amplification of stable lines expressing the protein. Protein for initial characterization was produced by transient expression from COS cells followed by Protein A purification.

As an example, a chimeric antibody producing cell line was generated by co-transfecting CHO DG44 cells with separate expression vectors for the heavy and light chains of the chimeric antibody, and the high copy number electroporation method was used to promote co-integration. (See, U.S. Patent 4,956,288). The chi220 heavy and light chains were cloned into the pD17 and pD16 expression vectors, respectively. Both vectors are derived from the InVitrogen plasmid pcDNA3, and contain the following features (Figure 12): (1) the neomycin resistance gene from pcDNA3 was replaced with the murine dihydrofolate reductase (DHFR) gene under control of the enhancerless SV40 promoter (also referred to as the "weakened DHFR"; note that only the promoter was weakened, not the DHFR enzyme - the enhancerless promoter still contains the SV40 origin of replication, so these vectors can be used in transient COS transfections); (2) the gene of interest is expressed from the CMV promoter, and the poly adenylation signal is from the bovine growth hormone gene; (3) the expression cassette for the gene of interest is flanked by transcription termination

sequences (i.e., 5' to the promoter and 3' to the poly A site); (4) the vectors contain two distinct restriction site polylinkers, one 3' to the promoter for cloning the gene of interest, and one 5' to the promoter for vector linearization prior to transfection; and (5) the ampicillin resistance gene and ColE1 origin for plasmid propagation in E. coli.

The heavy and light chain genes used were genomic constructs, with the following modifications: (1) the coding sequences for the heavy chain signal peptide, variable region and CH1 domain were contiguous (i.e., contained no introns); and (2) the coding sequences for the light chain signal peptide and variable region were contiguous.

Other expression vectors known by those skilled in the art, and capable of expressing a chimeric antibody of the present invention, are contemplated by the present invention. A nucleic acid sequence useful in an expression vector capable of expressing a heavy chain of a chimeric antibody of the present invention is shown in Figure 13; a nucleic acid sequence useful in an expression vector capable of expressing a light chain of a chimeric antibody of the present invention is shown in Figure 14.

The complete amino acid sequence of the heavy and light chains of the chimeric antibody ("chi220"), including the variable and constant regions, is as follows (the bold amino acids indicate variable heavy and variable light):

Heavy Chain Sequence (SEQ ID NO:3)

QIQLVQSGPE LKKPGETVRI SCKASGYAFT TTGMQWVQEM PGKGLKWIGW 50
INTHSGVPKY VEDFKGRFAF SLETSANTAY LQISNLKNED TATYFCVRSG 100
NGNYDLAYFA YWGQGTLVTV SAASTKGPSV FPLAPSSKST SGGTAALGCL 150
VKDYFPEPVT VSWNSGALTS GVHTFPAVLQ SSGLYSLSSV VTVPSSSLGT 200
QTYICNVNHK PSNTKVDKKV EPKSCDKTHT CPPCPAPELL GGPSVFLFPP 250
KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH NAKTKPREEQ 300
YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT ISKAKGQPRE 350
PQVYTLPPSR DELTKNQVSL TCLVKGFYPS DIAVEWESNG QPENNYKTTP 400
PVLDSDGSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH YTQKSLSLSP 450
GK 452

Light Chain Sequence (SEQ ID NO:4)

DIVLTQSPAT LSVTPGDRVS LSCRASQSIS DYLHWYQQKS HESPRLLIKY 50
ASHSISGIPS RFSGSGSGSD FTLSINSVEP EDVGIYYCQH GHSFPWTFGG 100
GTKLEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV 150

DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG LSSPVTKSFN RGEC 200 214

Several humanized forms of 220 were generated. This process involves the identification of murine and human germline sequences with the closest homology to the VH and VL domains. The murine germline sequences were used to identify likely locations of somatic mutations that have arisen during the process of affinity maturation. The human sequences were then used as template and regions of the sequence known or suspected to be important to the binding specificity are replaced in the human sequences for both VH and VL. The structures of these sequences were then modeled using as a template the protein with the closest homology for which a crystal structure has been solved. Plasmids encoding the humanized forms were generated using PCR directed mutagenesis and used to generate antibody by transient expression from COS cells. *In vitro* assays were performed with the chimeric and humanized antibodies of the present invention, and results are depicted in Figure 5. Figure 5a shows the results of a binding assay testing the binding of chi220 and h220v3 to hCD40-mG2b in an ELISA based assay. Wells of Immulon-2 plates were coated with hCD40-mG2b at a concentration of 10 ng/ml in PBS for 2 hrs. Wells were blocked with Specimen Diluent (Genetic Systems), and antibodies were added at the indicated concentrations. Following a 1hr incubation, wells were washed, and the presence of the antibody detected using peroxidase-conjugated goat anti-human IgG antibody. H220v3 is a humanized form of mAb 2.220. Values are the average of duplicate wells and error bars represent the standard deviation.

Figure 5b shows the results of an assay testing the inhibition of sgp39-mediated costimulation of human B cells with anti-human CD40 mAbs. Resting human tonsillar B cells (50,000/well) were incubated with sgp39 fusion protein, 20 μg/ml rabbit anti-human IgM coated immunobeads and the indicated concentrations of the anti-CD40 mAbs or medium only control in 96 well plates. 72 hrs after initiation of cultures, all wells were pulsed with 1 uCi/well [^{3}H]thymidine and the cells cultured for an additional 18 hrs. Cells were then harvested and incorporated [^{3}H]thymidine measured in a scintillation counter.

Based upon the results of *in vitro* assays (Figures 5a and 5b, that show both the chimeric and humanized antibody effectively bound CD40 and inhibited B cell stimulation) the chimeric antibody was chosen for further study.

Example 3

Efficacy of chi220

A. Chimeric mAb 2.220: Single-Dose Efficacy Study in Nonhuman Primates

Chi220 was evaluated in cynomolgus monkeys for its ability to suppress primary and secondary humoral immune responses to T cell-dependent antigens. In one study, groups of four monkeys were immunized with sheep erythrocytes (SRBCs) and given a secondary immunization of ovalbumin (OVA) immediately prior to receiving a single intravenous bolus dose of either chi220 at 10, 40, or 100 mg/kg or sterile phosphate buffered saline (PBS) as a control. Substantial suppression of the primary humoral immune response against SRBCs was observed at all three dose levels, demonstrating efficacy of chi220 in primates. A dose-dependent transient depletion of peripheral blood B cells was observed in all of the chi220-treated monkeys, with the time to recovery also being dose dependent. At the two highest doses, transient mild decreases in the group mean absolute numbers of peripheral blood T cells were observed. Transient minimal decreases in serum IgM levels were observed, with no drug related changes in serum levels of IgG or IgA.

To assess the induction of immunological tolerance and reversibility of immunosuppressive activity, all monkeys were immunized with OVA, SRBCs, and a neoantigen, keyhole limpet hemocyanin (KLH) on day 149, when serum levels of chi220 in the 100 mg/kg group were below levels believed to be immunosuppressive (~10 μg/ml) and the numbers of peripheral blood B cells had returned to predose levels. The anti-SRBC response at the lowest dose level was generally comparable to the primary anti-SRBC antibody response in the control monkeys. However, the antibody response to SRBCs was still partially or substantially suppressed in the monkeys treated at the two higher dose levels.

To further explore the dose dependence of immunosuppression and B cell depletion, a second study was performed in which additional monkeys (four/group) were immunized with SRBCs, and then given a single dose of chi220 at 0.1 or 1.0

mg/kg or PBS. Suboptimal immunosuppression of the antibody response to SRBCs was observed at both dose levels. Moderate depletion of peripheral blood B cells was evident in monkeys that received 1.0 mg/kg chi220 by Day 8, reversing by Day 29. At 0.1 mg/kg, a decrease in the mean number and percentage of peripheral blood B cells was observed, but values were not outside the normal historical ranges for percent B cells. Historical limits have not been established for absolute numbers of peripheral blood B cells. Transient minimal decreases in peripheral blood T cell numbers and mild decreases in *ex vivo* T cell proliferation were observed in monkeys that received 1 mg/kg chi220. Finally, there was no evidence of complement activation or drug-related changes in the serum levels of IL-6 or TNFα. *Ex vivo* T cell activation, complement activation, and serum cytokine levels were not assessed in monkeys treated with 10, 40, or 100 mg/kg chi220.

In both studies, serum samples were examined following chi220 administration for circulating levels of test article, and to assess antibody formation against the test article. Pharmacokinetic analysis indicated that the mean peak serum concentration (Cmax) of chi220 did not increase in a manner proportional to the dose increment, and that the half-life of chi220 became prolonged as the dose administered was increased. Chi220 was found to be immunogenic when administered at 0.1, 1 or 10 mg/kg. At circulating concentrations above 10 μg/ml, it appears that chi220 can suppress the antibody response directed against it.

1. Experimental Protocol

In the initial study mentioned above, cynomolgus monkeys were assigned to four groups consisting of two males and two females each. All monkeys were immunized 28 days prior to chi220 or control article administration with OVA (5 mg/kg, im and 10 mg/kg, sc). On Day 1, all monkeys were immunized with SRBCs (1.7 ml/kg of a 10% suspension, iv) and given a secondary immunization of OVA (5 mg/kg, im and 10 mg/kg, sc) immediately prior to receiving a single intravenous bolus dose of either chi220 at 10, 40, or 100 mg/kg or sterile PBS as a control. On Day 149, after the serum levels of chi220 had fallen below putatively immunosuppressive levels (~10 μg/ml) and the levels of peripheral blood B cells had returned to predose levels in all groups, the monkeys were immunized with OVA, SRBCs, and KLH (10

mg/animal, im). The purpose of the KLH immunization was to show that the monkeys were able to mount an immune response to a neoantigen after being treated with chi220.

In order to demonstrate a better dose response with respect to immunosuppression and peripheral blood B cell depletion, additional monkeys in a second study (two/sex/group) were immunized with SRBCs, and then given a single dose of either chi220 at 0.1 or 1.0 mg/kg or PBS as a control on Day 1. Hematological parameters and peripheral blood lymphocyte subpopulations were monitored at selected time points during both studies. Serum chemistry parameters were monitored in monkeys that received 10, 40, or 100 mg/kg chi220, but were not monitored at the 0.1 and 1 mg/kg dose levels because no drug-related findings were observed at the higher doses. In addition, serum levels of IgM, IgG, IgA, and chi220 were measured. To assess efficacy, specific IgM and IgG antibody formation against the SRBC and OVA immunogens was determined on the appropriate serum samples obtained just prior to immunogen administration and weekly thereafter. Specific IgM and IgG antibody formation against the test article for monkeys that received chi220 was determined prior to test article administration on Day 1, and weekly thereafter. Geometric mean titers were used when comparing antibody responses between groups. In addition, total hemolytic complement activity (CH_{50}) and C4d fragment levels were measured, and TNF-α and IL-6 levels were determined in monkeys that received 0.1 or 1 mg/kg chi220 at selected time points following chi220 administration. *Ex vivo* peripheral blood T cell activation was also assessed following stimulation with concanavalin A in monkeys receiving 0.1 and 1 mg/kg chi220 on Days 17 and 31 to assess the effects of chi220 on T cell responsiveness to a mitogen. Finally, all monkeys were observed daily for clinical signs of toxicity, body weights recorded weekly, and food consumption monitored daily.

Monkeys were immunized with SRBC prior to receiving vehicle or 10, 40, or 100 mg/kg chi220 (Figure 6a) or 0.1 or 1 mg/kg chi220 (Figure 6b) on Day 1. Serum samples were analyzed for IgM anti-SRBC antibodies by ELISA. Data are expressed as the geometric mean anti-SRBC antibody end-point titer (EPT) for each group (n=2 [100 mg/kg group beyond Day 15] or 4), where EPT is equivalent to the reciprocal of

the greatest dilution of serum with an absorbance of greater than two times the mean plate background.

2. Results

a. Anti-SRBC Antibody Response

When administered to monkeys at 10, 40, or 100 mg/kg, chi220 was effective at substantially suppressing the primary antibody response against SRBCs. On the peak day of the control primary IgM anti-SRBC antibody response (Day 8), the mean primary IgM anti-SRBC antibody response was suppressed approximately 92–94% in the monkeys treated with 10, 40, and 100 mg/kg chi220, compared to controls (Figure 6a). The group mean IgM anti-SRBC antibody response did not become positive through Day 85 at the 10, 40 or 100 mg/kg dose levels. On the peak day of the control primary IgG anti-SRBC antibody response (Day 15), the mean primary IgG anti-SRBC antibody response was suppressed 98%, 99%, and 85% in monkeys that received 10, 40, and 100 mg/kg, respectively, compared to controls (Figure 7a). Higher overall predose anti-SRBC antibody titers in the 100 mg/kg group may have accounted for the apparent lack of dose-dependent immunosuppression. Overall, monkeys treated with 10 or 100 mg/kg chi220 did not mount a primary IgG anti-SRBC antibody response through Day 85. However, two of the monkeys treated with 40 mg/kg chi220 had a delayed primary IgG antibody response to SRBCs (comparable to the control response in magnitude), which became positive by Day 36 and peaked on Day 51.

On Day 149, after the serum levels of chi220 had fallen below putatively immunosuppressive levels (~10 μg/ml) and the levels of peripheral blood B cells had returned to predose levels in all groups, the monkeys were immunized a second time with SRBCs. As expected, control monkeys mounted a strong secondary IgG antibody response to SRBCs. Monkeys treated with 10 mg/kg chi220 mounted primary IgM and IgG antibody responses to SRBCs that were generally comparable to the primary antibody response in the control monkeys. However, the antibody response to SRBCs was still partially suppressed at the 40 mg/kg dose level and substantially suppressed at the 100 mg/kg dose level. Although two monkeys treated with 40 mg/kg chi220 that had previously mounted weak primary antibody responses

to SRBCs developed IgM and IgG anti-SRBC antibody titers characteristic of a secondary antibody response, the anti-SRBC antibody responses in the two other monkeys in that group and the remaining monkeys treated with 100 mg/kg chi220 was still approximately 90% suppressed compared to the mean primary anti-SRBC antibody response of the control monkeys.

Suboptimal immunosuppression of the antibody response to SRBCs was observed following administration of 0.1 or 1.0 mg/kg chi220 (Figures 6b and 7b). While all of the chi220-treated monkeys mounted a positive IgM antibody response to the SRBC antigen, the overall mean peak IgM anti-SRBC antibody response was suppressed approximately 56% in the monkeys treated with 1 mg/kg chi220 compared to the mean peak control response. No suppression of the IgM anti-SRBC antibody response was observed in monkeys treated with 0.1 mg/kg chi220. The mean IgM anti-SRBC antibody response peaked on Day 15 in the control monkeys, and on Day 8 in the monkeys that received 0.1 and 1.0 mg/kg chi220. Overall, the mean peak IgG anti-SRBC antibody response was suppressed 56% and 42% in the monkeys treated with 0.1 and 1.0 mg/kg chi220, respectively. The mean IgG anti-SRBC antibody response peaked on Day 15 in the control monkeys and monkeys treated with 1 mg/kg chi220, and on Day 8 in the monkeys that received 0.1 mg/kg chi220.

b. Anti-OVA Antibody Response

Monkeys were administered an intravenous dose of 10, 40, or 100 mg/kg chi220 on Day 1. In addition all monkeys were immunized with OVA on Days -28, 1, and 149. Serum samples were analyzed for IgM (Figure 8a) or IgG (Figure 8b) anti-OVA antibodies. Data are expressed as the geometric mean anti-OVA endpoint titer (EPT) for each group (n=2 [100 mg/kg group beyond Day 15] or 4), where EPTs are equivalent to the reciprocal of the greatest dilution of serum with an absorbance of greater than two times the mean plate background.

Specific IgM and IgG antibody formation against OVA was monitored weekly during the study in monkeys that received 10, 40, or 100 mg/kg chi220. The primary and secondary anti-OVA antibody responses were highly variable and generally weak in all monkeys (Figure 8). Monkeys scheduled to receive chi220 on Day 1 had greater anti-OVA antibody titers than monkeys in the control group.

On Day 149, the monkeys were given a tertiary OVA immunization. All of the monkeys mounted positive IgG antibody responses to OVA within 7 days following challenge. Control monkeys and monkeys treated with 10 mg/kg chi220 had antibody titers characteristic of a tertiary antibody response, whereas monkeys treated with either 40 or 100 mg/kg chi220 developed antibody titers that were more characteristic of a secondary antibody response.

c. Anti-KLH Antibody Response

Monkeys were administered an intravenous dose of 10, 40, or 100 mg/kg chi220 on Day 1. In addition, all monkeys were immunized with KLH on Day 149. Serum samples were analyzed for IgM (Figure 9a) or IgG (Figure 9b) anti-KLH antibodies. Data are expressed as the geometric mean anti-KLH endpoint titer (EPT) for each group (n=2 [100 mg/kg group beyond Day 15] or 4), where EPTs are equivalent to the reciprocal of the greatest dilution of serum with an absorbance of greater than two times the mean plate background.

On Day 149, after the serum levels of chi220 had fallen below putatively immunosuppressive levels (~10 μg/ml) and the levels of peripheral blood B cells had returned to predose levels in all groups, the monkeys were immunized with KLH (10 mg/animal, im). All monkeys mounted positive IgM and IgG antibody responses to KLH, demonstrating that the ability to respond to a new antigen was not compromised (Figure 9).

d. Serum Levels of Test Article and Anti-Test Article Antibody Response

Serum samples were examined following chi220 administration to determine circulating levels of test article and to assess antibody formation against the test article. The mean peak serum concentration (Cmax) of chi220 occurred three minutes following the administration of 10 or 40 mg/kg doses and six hours following administration of the 100 mg/kg dose. Cmax values of chi220 were 329, 2429, and 2343 μg/ml in the monkeys treated with 10, 40, or 100 mg/kg chi220, respectively. There was, however, considerable variation in the Cmax of individual monkeys in the 40 and 100 mg/kg groups. The mean serum half-life of chi220 was estimated to be approximately 114, 173 and 315 hours in monkeys treated with 10, 40, or 100 mg/kg chi220, respectively.

Mean Cmax values, occurring three minutes following chi220 administration, were 1.77 and 33 μg/ml for 0.1 and 1 mg/kg doses, respectively. No gender related differences in the serum levels of chi220 were observed within each dose level. Mean AUC_{inf} values were 15.5 and 847 ug.h/ml, for 0.1 and 1 mg/kg doses, respectively. Taken together, the studies suggest that the half-life of chi220 becomes prolonged as the dose administered is increased. Furthermore, it appears that the Cmax of chi220 increases in a manner disproportionate to the dose increment.

Although the IgM anti-test article response was minimal or absent in the monkeys that received 10, 40, or 100 mg/kg chi220, a significant IgG anti-test article antibody response was observed in the monkeys that received 10 mg/kg chi220. The mean IgG anti-test article antibody response in the monkeys that received 10 mg/kg chi220 became positive on Day 29, approximately 1 week after the mean group serum concentration of chi220 had fallen below 10 μg/ml, and peaked on Days 36 and 43 at a geometric mean titer of 12,627. The appearance of IgG anti-test article antibodies in the monkeys that were treated with 10 mg/kg chi220 also coincided with the first detectable increases in B cell numbers following depletion. By the last day measured (Day 149), the monkeys that received 40 or 100 mg/kg chi220 had still not mounted a positive antibody response against chi220, although the group mean chi220 serum levels were below 10 μg/ml by Day 57 (40 mg/kg group) or Day 92 (100 mg/kg group).

Chi220 was immunogenic when administered at 0.1 or 1 mg/kg. Three of four monkeys that received either 0.1 or 1 mg/kg chi220 had weakly positive IgM anti-test article antibody responses by Day 15 during the study. Three of four monkeys treated with 1 mg/kg chi220 had significant IgG anti-test article antibody responses by Day 22, peaking at a geometric mean endpoint titer of 16,618. Overall, the geometric mean IgG anti-test article antibody response was not positive in the monkeys that received 0.1 mg/kg chi220, and only one monkey that received 0.1 mg/kg chi220 had a weakly positive IgG anti-test article antibody response, peaking at an endpoint titer of 2430 on Day 22. Collectively, these data suggest that chi220 is capable of immunosuppressing an antibody response against itself at serum levels of greater than approximately 10 μg/ml.

Example 4

Generation of Humanized Anti-CD40 Antibodies F4 and L3.17

A variety of methods known in the art have been used for the humanization of mAbs. Structure-based approaches have proven useful but the complexity that arises from the large number of framework residues potentially involved in binding activity diminishes the rate of success. Rather than predicting the optimal framework based on modeling, the antibody library approach described below permits identification of active framework conformations based on screening numerous combinations. Mutagenesis approaches coupled to selection methods permit the analysis of many variants and mimics the *in vivo* maturation process (reviewed in Marks, J.D., et al., (1992) *J. Biol. Chem.* 267:16007-16010). Codon-based mutagenesis permits the construction of libraries that characterize the contribution of specific residues and thus, is more efficient than random mutagenesis approaches. For example, error-prone PCR can not be used to synthesize the combinatorial framework libraries described below. Moreover, random mutagenesis creates larger more diverse libraries and unfortunately, the majority of mutations do not enhance the binding activity of the mAb. Consequently, larger numbers of clones must be screened to identify active variants.

A strategy termed "guided selection" has been used to isolate human mAbs from a phage display library in a two-step process that uses a rodent mAb as a template (Jespers, L. S., et al., (1994) *Bio/Technology* 12:899-903). Recently, a variation of guided selection using phage display technologies was described in which a chimeric Fd fragment was used to select a L chain from a library containing human L chains with grafted murine CDR3 (Rader, C., et al., (1998) *Proc. Natl. Acad. Sci. USA* 95:8910-8915). Subsequently, the most active L chain was used to select an H chain from a human H chain library containing the murine HCDR3. The mAbs isolated by these approaches are entirely human (Jespers, *supra*) or mostly human (Rader, *supra*), but the large antibody diversity introduced at each step of the processes necessitates the use of affinity enrichment methods.

The following materials and methods were utilized to generate the humanized anti-CD40 antibodies F4 and L3.17 of the present invention.

1. Construction of Chimeric anti-CD40

Based on the sequence of anti-CD40 murine mAb 2.220 overlapping oligonucleotides encoding V_H and V_L (69-75 bases in length) were synthesized and purified. The variable H and L domains were synthesized separately by combining 25 pmol of each of the overlapping oligonucleotides with *Pfu* DNA polymerase (Stratagene) in a 50 µl PCR reaction consisting of 5 cycles of: denaturing at 94°C for 20 sec, annealing at 50°C for 30 sec, ramping to 72°C over 1 min, and maintaining at 72°C for 30 sec. Subsequently, the annealing temperature was increased to 55°C for 25 cycles. A reverse primer and a biotinylated forward primer were used to further amplify 1 µl of the fusion product in a 100 µl PCR reaction using the same program. The products were purified by agarose gel electrophoresis, electroeluted, and phosphorylated by T4 polynucleotide kinase (Boehringer Mannheim) and were then incubated with streptavidin magnetic beads (Boehringer Mannheim) in 5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA, 1 M NaCl, and 0.05% Tween 20 for 15 min at 25°C. The beads were washed and the non-biotinylated, minus strand DNA was eluted by incubating with 0.15 M NaOH at 25°C for 10 min. Chimeric anti-CD40 Fab was synthesized in a modified M13IX104 vector (Kristensson, K., et al., (1995) *Vaccines 95*, pp. 39-43, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), termed M13IX104CS, by hybridization mutagenesis (Rosok, M. J., et al., (1996) *J. Biol. Chem.* 271:22611-22618; Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492) using the V_H and V_L oligonucleotides in 3-fold molar excess of the uridinylated vector template. The M13IX104 vector was modified by replacing cysteine residues at the end of the kappa and γ1 constant regions with serine. The reaction was electroporated into DH10B cells and titered onto a lawn of XL-1 Blue.

2. Construction of Combinatorial Framework and Framework/CDR3 Libraries.

The combinatorial framework library (Hu I) was synthesized by the same method used to construct the chimeric anti-CD40, with modifications. Overlapping

oligonucleotides encoding the framework regions of the H and L variable domains of the human template and the murine anti-CD40 CDRs as defined by Kabat *et al.* (Kabat, E.A., et al., (1991) Sequences of proteins of immunological interest (5th Ed), Washington DC: United States Department of Health and Human Services; Kabat, E.A., et al., (1977) *J. Biol. Chem.* 252:6609-6616) were synthesized. Degenerate oligonucleotides encoding both the murine and the human amino acids at seven V_H and one V_K framework position were synthesized (Figure 15, residues marked with asterisk).

The framework/HCDR3 (Hu II) and framework/HCDR3/LCDR3 (Hu III) libraries were synthesized by the same method as the combinatorial framework library, with modifications. The CDR residues selected for mutagenesis were: Ser^{95}-Tyr^{102} in HCDR3 and Gln^{89}-Thr^{97} in LCDR3 (Figure 15, underlined). Oligonucleotides encoding HCDR3 and LCDR3 were designed to mutate a single CDR residue and were synthesized by introducing NN(G/T) at each position as described in the art (Glaser, S. M., et al., (1992) *J. Immunol.* 149:3903-3913). The overlapping oligonucleotides encoding the framework library and non-library murine CDRs were combined with 25 pmol of the oligonucleotides encoding mutated HCDR3 or with 25 pmol each of the oligonucleotides encoding mutated HCDR3 and LCDR3.

3. Screening of Phage Expression Libraries

The Hu II and Hu III libraries were initially screened by a modified plaque lift approach known in the art, termed capture lift (Watkins, J. D., et al., (1998) *Anal. Biochem.* 256:169-177). Briefly, nitrocellulose filters (82-mm) were coated with goat anti-human kappa, blocked with 1% BSA, and were applied to an agar plate containing the phage-infected bacterial lawn. In the initial screen, phage were plated at 10^5 phage/100-mm plate. After the capture of phage-expressed anti-CD40 variant Fabs, the filters were incubated 3 h at 25°C with 5 ng/ml CD40-Ig in PBS containing 1% BSA. The filters were rinsed four times with PBS containing 0.1% Tween 20 and were incubated with goat anti-mouse IgG_{2b}-alkaline phosphatase conjugate (Southern

Biotechnology) diluted 3000-fold in PBS containing 1% BSA for 1 h at 25°C. The filters were washed four times with PBS containing 0.1% Tween 20 and were developed as described (Watkins (1998), *supra*). To isolate individual clones, positive plaques from the initial screen were picked, replated at lower density (<10^3 phage/100-mm plate), and were screened by the same approach.

The Hu I combinatorial library was first screened by an ELISA that permits the rapid assessment of the relative affinities of the variants (Watkins, J. D., et al., (1997) *Anal. Biochem.* 253:37-45). In addition, the ELISA was used to characterize clones identified by capture lift screening. Briefly, microtiter plates were coated with 5 µg/ml goat anti-human kappa (Southern Biotechnology) and blocked with 3% BSA in PBS. Next, 50 µl Fab from the *Escherichia coli* culture supernatant or from the cell lysate, was incubated with the plate 1 h at 25°C, the plate was washed three times with PBS containing 0.1% Tween 20, and 0.1 µg/ml CD40-Ig in PBS containing 1% BSA for 2 h at 25°C. The plate was washed three times with PBS containing 0.1% Tween 20 and goat anti-mouse IgG_{2b}-alkaline phosphatase conjugate diluted 3000-fold in PBS containing 1% BSA was added for 1 h at 25°C. The plate was washed three times with PBS containing 0.1% Tween 20 and was developed as described in the art (Watkins (1997), *supra*).

4. DNA Sequencing

Single-stranded DNA was isolated and the H and L chain variable region genes of the humanized antibodies of the present invention were sequenced by the fluorescent dideoxynucleotide termination method (Perkin-Elmer, Foster City, CA).

The nucleic acid (SEQ ID NO:7) and amino acid (SEQ ID NO:8) sequence of the variable light chain of humanized antibody F4 is as follows:

```
GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT   42
 E   I   V   L   T   Q   S   P   A   T   L   S   L   S   14

CCA GGG GAA AGA GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT   84
 P   G   E   R   A   T   L   S   C   R   A   S   Q   S   28

ATT AGC GAT TAC TTA CAT TGG TAC CAA CAG AAA CCT GGC CAG  126
 I   S   D   Y   L   H   W   Y   Q   Q   K   P   G   Q   42

GCT CCC AGG CTC CTC ATC TAT TAC GCA TCC CAC TCC ATC TCT  168
```

```
  A   P   R   L   L   I   Y   Y   A   S   H   S   I   S   56

GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC 210
 G   I   P   A   R   F   S   G   S   G   S   G   T   D  70

TTC ACT CTC ACC ATC AGC AGC CTA GAG CCT GAA GAT TTT GCA 252
 F   T   L   T   I   S   S   L   E   P   E   D   F   A  84

GTT TAT TAC TGT CAG CAT GGC CAC TCT TTT CCT TGG ACC TTC 294
 V   Y   Y   C   Q   H   G   H   S   F   P   W   T   F  98

GGA GGG GGG ACC AAG GTG GAA ATT AAA                     321
 G   G   G   T   K   V   E   I   K                     107
```

The nucleic acid (SEQ ID NO:9) and amino acid (SEQ ID NO:10) sequence of the variable heavy chain of humanized antibodies F4 and L3.17 is as follows:

```
CAG GTG CAG CTG GTG CAA TCT GGG TCT GAG TTG AAG AAG CCT  42
 Q   V   Q   L   V   Q   S   G   S   E   L   K   K   P  14

GGG GCC TCA GTG AAG GTT TCC TGC AAG GCT TCT GGA TAC GCC  84
 G   A   S   V   K   V   S   C   K   A   S   G   Y   A  28

TTC ACT ACC ACT GGC ATG CAG TGG GTG CGA CAG GCC CCT GGA 126
 F   T   T   T   G   M   Q   W   V   R   Q   A   P   G  42

CAA GGG CTT GAG TGG ATG GGA TGG ATC AAC ACC CAC AGC GGG 168
 Q   G   L   E   W   M   G   W   I   N   T   H   S   G  56

GTC CCA AAG TAT GTC GAG GAC TTC AAA GGA CGG TTT GTC TTC 210
 V   P   K   Y   V   E   D   F   K   G   R   F   V   F  70

TCC TTG GAC ACC TCT GTC AGC ACG GCA TAT CTG CAG ATC AGC 252
 S   L   D   T   S   V   S   T   A   Y   L   Q   I   S  84

AGC CTA AAG GCT GAG GAC ACT GCC GTG TAT TAC TGT GCG AGA 294
 S   L   K   A   E   D   T   A   V   Y   Y   C   A   R  98

TCT GGC AAT GGG AAC TAT GAC CTG GCA TAC TTT AAG TAT TGG 336
 S   G   N   G   N   Y   D   L   A   Y   F   K   Y   W 112

GGC CAG GGA ACC CTG GTC ACC GTC TCC TCA                 366
 G   Q   G   T   L   V   T   V   S   S                 122
```

The nucleic acid (SEQ ID NO:11) and amino acid (SEQ ID NO:12) sequence of the variable light chain of humanized antibody L3.17 is as follows:

```
GAA ATT GTG TTG ACA CAG TCT CCA GCC ACC CTG TCT TTG TCT  42
 E   I   V   L   T   Q   S   P   A   T   L   S   L   S  14

CCA GGG GAA AGA GCC ACC CTC TCC TGC AGG GCC AGT CAG AGT  84
 P   G   E   R   A   T   L   S   C   R   A   S   Q   S  28
```

```
ATT AGC GAT TAC TTA CAT TGG TAC CAA CAG AAA CCT GGC CAG 126
 I   S   D   Y   L   H   W   Y   Q   Q   K   P   G   Q  42

GCT CCC AGG CTC CTC ATC TAT TAC GCA TCC CAC TCC ATC TCT 168
 A   P   R   L   L   I   Y   Y   A   S   H   S   I   S  56

GGC ATC CCA GCC AGG TTC AGT GGC AGT GGG TCT GGG ACA GAC 210
 G   I   P   A   R   F   S   G   S   G   S   G   T   D  70

TTC ACT CTC ACC ACT AGC AGC CTA GAG CCT GAA GAT TTT GCA 252
 F   T   L   T   I   S   S   L   E   P   E   D   F   A  84

GTT TAT TAC TGT CAG CAT GGC CAC TCT TAT CCT TGG ACC TTC 294
 V   Y   Y   C   Q   H   G   H   S   Y   P   W   T   F  98

GGA GGG GGG ACC AAG GTG GAA ATT AAA                     321
 G   G   G   T   K   V   E   I   K                     107
```

5. Expression and Purification of Fab

Certain Fabs were cloned into an expression vector under the control of the arabinose-regulated BAD promoter. In addition, a six-histidine tag was fused to the carboxyl-terminus of the H chain to permit purification with nickel-chelating resins. Purified Fab was quantitated as described (Watkins (1997), *supra*).

6. Characterization Assays

Immulon II microtiter plates were coated with 0.1 μg/ml CD40-Ig in PBS for 16 h at 4°C and were blocked with 3% BSA in PBS. The plates were washed three times in PBS containing 0.1% Tween 20 and Fab released from periplasmic space was diluted serially three-fold in PBS containing 1% BSA and incubated with the plate 2 h at 25°C. Subsequently, the plate was washed four times with PBS containing 0.1% Tween 20 and binding of antibody was detected by incubating with goat anti-human kappa-alkaline phosphatase conjugate diluted 2000-fold in PBS containing 1% BSA for 1 h at 25° C. The plate was washed four times with PBS containing 0.1% Tween 20 and was developed colorimetrically (Watkins (1997), *supra*).

To test the variants for inhibition of ligand binding, Immulon II microtiter plates were coated with 2 μg/ml anti-murine CD8 to capture sgp39 fusion protein which expresses the CD8 domain. The plates were rinsed once with PBS containing 0.05% Tween 20, and were blocked with 3% BSA in PBS. The plate was washed

once with PBS containing 0.05% Tween 20 and was incubated with cell culture media containing saturating levels of sgp39 for 2 h at 25°C. Unbound sgp39 was aspirated and the plate was washed two times with PBS containing 0.05% Tween 20. Next, 25 µl of purified variant Fabs diluted serially 3-fold in PBS was added followed by 25 µl of 4 µg/ml CD40-human Ig in PBS. The plates were incubated 2 h at 25°C and were washed three times with PBS containing 0.05% Tween 20. Bound CD40-Ig was detected following a 1 h incubation at 25°C with goat $F(ab')_2$ anti-human IgG Fcγ-specific horseradish peroxidase conjugate (Jackson) diluted 10,000-fold in PBS. The plate was washed four times with PBS containing 0.05% Tween 20 and binding was quantitated colorimetrically by incubating with 1 mg/ml *o*-phenylenediamine dihydrochloride and 0.003% hydrogen peroxide in 50 mM citric acid, 100 mM Na_2HPO_4, pH 5. The reaction was terminated by the addition of H_2SO_4 to a final concentration of 0.36 M and the absorbance at 490 nm was determined.

7. BIAcore Analysis

The kinetic constants for the interaction between CD40 and the anti-CD40 variants were determined by surface plasmon resonance (BIAcore). CD40-Ig fusion protein was immobilized to a (1-ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride) and *N*-hydroxysuccinimide-activated sensor chip CM5 by injecting 8 µl of 10 µg/ml CD40-Ig in 10 mM sodium acetate, pH 4. CD40-Ig was immobilized at a low density (~150 RU) to prevent rebinding of Fabs during the dissociation phase. To obtain association rate constants (k_{on}), the binding rate at six different Fab concentrations ranging from 25-600 nM in PBS was determined at a flow rate of 20 µl/min. Dissociation rate constants (k_{off}) were the average of six measurements obtained by analyzing the dissociation phase. Sensorgrams were analyzed with the BIAevaluation 3.0 program. K_d was calculated from $K_d = k_{off}/k_{on}$. Residual Fab was removed after each measurement by prolonged dissociation.

The results of kinetics analysis for the humanized antibodies F4 and L3.17 compared to a chimeric Fab are shown in Table 1 below:

Table 1

Clone ID#	k_{on}	K_{off}	K_d	Comment
Chimeric Fab	8.43E+5	2.65E-3	3.14 nM	Prepared by papain cleavage of chimeric 2.220 IgG
F4	2.00E+6	4.77E-4	0.24 nM	Humanized
L3.17	3.17E+6	3.28E-4	0.10 nM	Humanized

8. Humanization Results

As discussed above, the murine anti-CD40 mAb variable region framework sequences were used to identify the most homologous human germline sequences. The H chain framework residues were 74% identical to human germline VH7 (7-4.1) and JH4 sequences while the L chain was 75% identical to the corresponding human germline VKIII (L6) and JK4 sequences. Alignment of the H and L chain variable sequences is shown in Figure 15. CDR residues, as defined by Kabat *et al.* (Kabat, E.A., et al., (1991) Sequences of proteins of immunological interest (5th Ed), Washington DC: United States Department of Health and Human Services; Kabat, E.A., et al., (1977) *J. Biol. Chem.* 252:6609-6616) are underlined and were excluded from the homology analysis. Framework residues that differed between the murine mAb and the human templates were assessed individually.

Based on structural and sequence analysis, antibody CDRs with the exception of HCDR3 display a limited number of main chain conformations termed canonical structures (Chothia, C. et al., (1987) *J. Mol. Biol.* 196:901-917; Chothia, C., et al., (1989) *Nature* 342:877-883). Moreover, certain residues critical for determining the main chain conformation of the CDR loops have been identified (Chothia (1987), *supra*; Chothia (1989), *supra*). Canonical framework residues of murine anti-CD40 were identified therefore, and it was determined that amino acids at all critical canonical positions within the H and L chain frameworks of the human templates were identical to the corresponding murine residues.

Surface-exposed murine amino acids not normally found in human antibodies are likely to contribute to the immunogenicity of the humanized mAb (Padlan, E. A. (1991) *Mol. Immunol.* 28:489-498). Therefore, framework residues differing

between murine anti-CD40 and the human templates were analyzed and based on solvent exposure were predicted to be buried or located on the surface of the antibody (Padlan (1991), *supra*). Solvent-exposed framework residues distal to the CDRs were not expected to contribute to antigen binding significantly and thus, with the exception of two H chain residues all were changed to the corresponding human amino acid to decrease potential immunogenicity. H chain residues 28 and 46 were predicted to be solvent exposed. However, H28 is located within the HCDR1 region as defined by Chothia et al., *supra,* and potentially interacts with the antigen. In addition, the lysine at H46 in the murine mAb is somewhat unusual and significantly different from the glutamic acid of the human template. Therefore, the murine and human residues at H28 and H46 were expressed in the combinatorial library (Figure 15, asterisks).

The remaining differing framework residues, all predicted to be mostly buried within the antibody, were evaluated for: (1) proximity to CDRs; (2) potential to contact the opposite domain in the V_K-V_H interface; (3) relatedness of the differing amino acids; and (4) predicted importance in modulating CDR activity as defined by Studnicka et al. (Studnicka, G. M., et al. (1994) *Protein Eng.* 7:805-814). The majority of L chain framework differences in buried residues were related amino acids at positions considered not likely to be directly involved in the conformation of the CDR. However, L49 is located adjacent to LCDR2, potentially contacts the V_H domain, is unrelated to the human residue, and may be involved in determining the conformation of LCDR2. For these reasons, the murine and human amino acids at L49 were both expressed in the combinatorial framework library (Figure 15, asterisk).

Analysis of the murine H chain sequence and the human template was more complex. Residue H9 is a proline in the murine mAb while the human template contains an unrelated serine residue. Position H9 may also play a role in modulating the conformation of the CDR and thus, was selected as a combinatorial library site (Figure 15, asterisks). The remaining buried framework residues that differed between murine anti-CD40 and the H chain template were at framework positions 38, 39, 48, and 91. Murine anti-CD40 mAb contained glutamine and glutamic acid at H38 and H39, respectively, while the human template contained arginine and

glutamine. Residue H38 is in proximity to the HCDR1, the glutamine→arginine change is non-conserved, and expression of glutamine at this site in murine Abs is somewhat unusual. Similarly, glutamic acid→glutamine is a non-conservative difference for buried amino acids, H39 is a potential V_K contact residue, and glutamic acid is somewhat unusual in murine mAbs. Residue H48 is in close proximity to HCDR2 and H91 is predicted to be a high risk site (Studnicka (1994), *supra*; Harris, L. et al., (1995) *Prot. Sci.* 4:306-310) that potentially contacts the V_K domain. Thus, both murine and human residues were expressed at H38, 39, 48, and 91 (Figure 15, asterisks).

In summary, the framework library consisted of murine CDRs grafted into the human templates. In addition, one framework residue on the L chain and seven framework residues on the H chain were deemed potentially important for maintaining the activity of the mAb. All of these sites were characterized by synthesizing a combinatorial library that expressed all possible combinations of the murine and human amino acids found at these residues. The total diversity of this library, termed Hu I, was 2^8 or 256 variants (Table 2 below).

Table 2: Summary of phage-expressed anti-CD40 antibody libraries.

Library	Library Positions	Size*	Screened†
Hu I	Framework	256	2.4×10^3
Hu II	framework, HCDR3	1.1×10^5	2.0×10^6
Hu III	framework, HCDR3, LCDR3	3.1×10^7	5.5×10^5

*Number of unique clones based on DNA sequence. Thirty-two codons are used to encode all 20 amino acids at each CDR position.

†The Hu I library was screened by ELISA using antibodies expressed in small-scale bacterial cultures (Watkins (1997), *supra*). The Hu II and Hu III libraries were plated on XL-1 Blue/agar lawns at 10^5 plaques per 100-mm dish and were screened by capture lift (Watkins (1998), *supra*).

The Hu I library was expressed in small-scale (<1 ml) bacterial cultures, uniform quantities of Fab released from the periplasmic space were captured in a

microtiter plate, and the binding activity of the antibodies was compared directly by ELISA (Watkins (1997), *supra*). Although variants that bind the target antigen with affinities comparable to, or better than, the chimeric Fab were identified, the majority of Hu I clones screened were less active than the chimeric anti-CD40 Fab. Approximately 6% of randomly selected Hu I variants displayed binding activities comparable to the chimeric Fab (data not shown). The identification of multiple Hu I variants with activity comparable to the chimeric CD40 is consistent with the interpretation that the most critical framework residues were included in the combinatorial library.

Active clones were characterized further by titration on immobilized antigen, confirming the identification of multiple variants with enhanced affinity. For example, clone 19C11 binds the CD40 receptor with higher affinity than the chimeric Fab, as demonstrated by the shift in the titration profile (Figure 16, open circles vs. filled circles). DNA sequencing of 34 of the most active clones led to the identification of 24 unique framework combinations, each containing 2-6 murine framework residues (data not shown).

LCDR3 and HCDR3 contact antigen directly, interact with the other CDRs, and often affect the affinity and specificity of antibodies significantly (Wilson, I.A., et al., (1993) *Curr. Opin. Struct. Biol.* 3:113-118; Padlan, E.A. (1994) *Mol. Immunol.* 31:169-217). In addition, the conformation of LCDR3 and HCDR3 are determined in part by certain framework residues. To identify the most active antibody, codon-based mutagenesis (Glaser, S. M., et al., (1992) *J. Immunol.* 149:3903-3913) was used to synthesize oligonucleotides that introduce mutations at every position in HCDR3, one at a time, resulting in the expression of all 20 amino acids at each CDR residue. Each oligonucleotide encoded no more than a single amino acid alteration. The pool of oligonucleotides encoding the HCDR3 library was mixed with the overlapping oligonucleotides encoding the combinatorial framework and other CDRs to generate a framework/HCDR3 library. The diversity of this library, termed Hu II, was 1.1×10^5 (Table 2, above). A library for LCDR3 was synthesized in a similar manner. Oligonucleotides encoding the LCDR3, HCDR3, and the combinatorial framework were used to create a framework/HCDR3/LCDR3 iibrary, termed Hu III. The large

number of framework/CDR3 combinations resulted in a library with a complexity of 3.1×10^7 (Table 2, above).

Combining mutations in LCDR3 and/or HCDR3 with the framework library increased the potential diversity of humanized anti-CD40 variants from 256 to greater than 10^7. In order to screen these larger libraries more efficiently a modified plaque lift assay, termed capture lift, was used (Watkins (1998), *supra*). Briefly, phage-infected bacteria were plated on solid agar lawns and subsequently, were overlaid with nitrocellulose filters that had been coated with a Fab-specific reagent. Following the capture of nearly uniform quantities of phage-expressed Fab the filters were probed with 5 ng/ml CD40-Ig fusion protein. Because the filters were probed with antigen at a concentration substantially below the *K*d of the Fab, only variants displaying enhanced affinity were detectable. Multiple clones displaying higher affinities were identified following the screening of $>10^6$ variants from Hu II and $>10^5$ variants from the Hu III library using 82-mm filters containing $\approx 10^5$ variants per filter (Table 2).

Because of the high phage density on the filters, positive plaques were picked, replated at a lower density, and screened again. Subsequently, the variants producing the most intense colorimetric signal in the capture lift assay were further characterized by ELISA. As expected, the majority of clones identified by capture lift screening bound CD40 better than the chimeric Fab. Titration of the variants on immobilized CD40-Ig identified multiple clones displaying affinities greater than the chimeric and humanized Fab (Figure 16, compare open squares and filled triangles with circles).

The framework/CDR mutations that conferred enhanced affinity were identified by DNA sequencing. Unique variable region sequences were identified in 10/13 Hu II variants and 3/4 Hu III variants. Both the Hu II and Hu III variants contained 1-5 murine framework residues and 0-2 CDR3 mutations, as summarized in Table 3 below.

Table 3. Simultaneous optimization of framework and CDR residues identifies higher affinity variants.

Library	Clone	Murine Framework Residues*	CDR Mutations
	chimeric	(43)	0
Hu I	19C11	(2) H28, 48	0
Hu II	CW43	(3) H9, 28, 91	HCDR3, ^{101}A→R
	2B12	(5) H9, 28, 38, 46, 48	HCDR3, ^{101}A→K
Hu III	2B12	(5) H9, 28, 38, 46, 48	HCDR3, ^{101}A→K
	2B8	(1) H28	HCDR3, ^{101}A→K; LCDR3, ^{96}R→Y

*Number of murine framework residues that differ from the most homologous human germline sequence based on definition of CDRs of Kabat et al., *supra*. The number of murine framework residues differing from the human template is indicated in parentheses. All of the framework differences between the murine mAb and the humanized versions are located on the H chain (H) at the indicated positions using the numbering system of Kabat et al.

The affinities of bacterially-expressed chimeric Fab and select variants from each of the libraries were characterized more thoroughly using surface plasmon resonance measurements to determine the association and dissociation rates of purified Fab with immobilized CD40-Ig. Chimeric anti-CD40 had a dissociation constant K_d = 3.14 nM and, consistent with the screening results, many of the variants displayed higher affinities. Two of the best clones, F4 and L3.17, had *Kd* of 0.24 nM and 0.10 nM, respectively (Table 1). The improved affinities of the anti-CD40 variants were predominantly the result of slower dissociation rates as the association rates were very similar for all of the variants (ranging from 0.9 to 3.2 x 10^6 $M^{-1}s^{-1}$).

Finally, the variants displaying enhanced affinity were tested for their ability to block the binding of gp39 ligand to the CD40 receptor. The variants all inhibited the binding of soluble CD40-Ig fusion protein to immobilized gp39 antigen in a dose-dependent manner that correlated with the affinity of the Fabs (Figure 17). For example, the most potent inhibitor of ligand binding to CD40-Ig fusion protein was variant 2B8, which was also the variant with the highest affinity for CD40 (Figure

17). Variant 2B8 displayed ≈17-fold higher affinity for CD40 than did the chimeric Fab and inhibited ligand binding ≈7-fold more effectively.

Example 5

Mouse Model System

Applicants also developed and tested *in vivo* a rat anti-murine CD40 mAb designated 7E1-G2b and its predecessor, 7E1-G1. The generation of this antibody was performed in order to explore the potential of anti-CD40 therapy in murine models of autoimmune, inflammatory and transplant disease. The primary objective of the mouse model system was to generate an anti-murine counterpart that mimicked 2.220's complete and potent blockade of gp39/CD40 interaction while possessing weak costimulatory activity, and test it *in vivo* in standard experimental disease models.

A. Isolation and Characterization of Anti-Murine CD40 Monoclonal Antibodies 7E1-G1 and 7E1-G2b

1. Immunization, Fusion and Characterization

A recombinant murine CD40 immunoglobulin fusion protein consisting of the extracellular region of mouse CD40 fused to the hinge, CH2 and CH3 domains of a mouse IgG2a antibody (mCD40-mIg) was used to immunize an 8 week old female Lewis rat via footpad inoculation. Three days following the last immunization, leukocytes from the draining lymph nodes were fused with X63-Ag8.653 mouse myeloma cells to create rat x mouse heterohybridomas. Wells containing antibody specific for native mouse CD40 were identified for reactivity with the original mCD40-mIg immunogen by ELISA, and for reactivity with a CD40 positive mouse B cell lymphoma cell line (WEHI-231, ATCC CRL-1702). Supernatants were then tested for the ability to inhibit the binding of mCD40-mIg to soluble, recombinant mCD8-murine gp39 fusion protein, mgp39, the murine equivalent of sgp39. Approximately twelve of the most potent inhibitor master wells were cloned by a limiting dilution method.

Following cloning, functional assays were performed with culture supernatants and purified antibody in order to more accurately assess the ability of the anti-CD40

mAbs to inhibit the interaction of murine gp39 with CD40 and to determine their stimulatory properties. Inhibitory properties were measured by the ability to inhibit the binding of mgp39 to WEHI-231 using standard procedures known in the art. Stimulatory properties were measured by the induction of tight, homotypic adhesion of WEHI-231 cells and the proliferation of splenic B cells in the presence of the antibody and anti-IgM using procedures known in the art. From these results, three mAbs (5A3, 7E1-G1 and 8E1) were determined to be most like the anti-human CD40 mAb 2.220 with respect to gp39/CD40 blockade and level of costimulatory activity.

2. Selection of 7E1 as the Lead Anti-Murine CD40 mAb

In vivo studies in mice were aimed at identifying which of the blocking/non-stimulatory anti-CD40 mAbs most potently suppressed specific antibody responses to a T-dependent antigen. Suppression of the IgG antibody response to SRBCs in mice with anti-murine CD40 mAb was studied. Groups of five BALB/c mice were immunized IV with 1 x 10^8 SRBCs and concurrently treated ip with 1 mg of anti-murine CD40 mAbs 5A3, 7E1-G1 or 8E1. As controls, groups of similarly immunized mice were treated with MR1 (hamster anti-murine gp39, positive control, 250 ug), 6E9 (rat anti-human gp39, negative control, 1 mg) or PBS. Mice were evaluated for IgG anti-SRBC titers by ELISA on days 7, 14, 21 and 35. The results indicated that when administered as a single dose of antibody at the time of antigen challenge with SRBCs, mAb 7E1-G1 was shown to be a more effective suppressor of the IgG anti-SRBC response compared to mAbs 5A3 or 8E1, and was therefore selected as the lead anti-CD40 mAb for murine studies.

3. Isotype Switch Variant of mAb 7E1-G1

7E1-G1 did not possess effector function characteristics comparable to that of the chimeric 2.220 anti-human CD40 mAb (i.e., rat IgG1 is not as efficient as human IgG1 at complement fixation and Fc receptor interaction) and the profile of specific antibody suppression *in vivo* for 7E1 was not as complete as that seen with the 2.220 mAb in primates. Thus, an antibody having 7E1 specificity but with a rat isotype more like human IgG1 in its effector capabilities was sought. To this end, a natural isotype switch variant of 7E1, from an IgG1 to an IgG2b, was generated by the sib-selection technique (Hale et al., J. Immunol. Methods (1987) 103(1):59-67). Briefly,

an anti-CD40 mAb of the IgG2b isotype was identified by ELISA among supernatants of 96 well plates that had been seeded at 1000 cells/well with the original 7E1 hybridoma. Subsequent rounds of plating and identification of IgG2b positive wells at seeding densities of 200 and then 20 cells/well followed by two rounds of cloning by limiting dilution led to the isolation of a clonal IgG2b switch variant of 7E1, 7E1-G2b.

7E1-G2b is a legitimate switch variant of the IgG1 as demonstrated by three sets of data. First, N-terminal sequencing of the heavy chain showed that both versions were identical for the first 35 amino acid residues. Second, PCR analysis using primers specific for the variable heavy chain CDRs of 7E1-G1 yielded a band of appropriate size from cDNA obtained from either 7E1-G1 or 7E1-G2b, and not two other unrelated antibodies. Lastly, assessment of binding activity of purified lots of the two versions to immobilized mCD40-hIg in an ELISA using an anti-kappa tracer reagent yielded essentially identical titration curves.

B. *In Vivo* Studies

1. *In Vivo* Comparison of 7E1-G1 to 7E1-G2b in Antibody Response Model

7E1-G1 was compared to 7E1-G2b for efficacy *in vivo* using SRBC's as the T cell dependent antigen. Groups of three to five animals were immunized iv with SRBC and concurrently treated ip with the antibody 7E1-G1 or 7E1-G2b, at 1, 0.25, or 0.1 mg of compound on day 0 as indicated in Figure 10. Anti-murine gp39 mAb MR1 served as a positive control for immunosuppressive effect. MAb 6E9 and PBS served as irrelevant mAb and no mAb controls, respectively. Mice were evaluated for anti-SRBC titers by ELISA on days 7, 14 and 21. Titer represents the calculated dilution of serum to yield an OD value =0.3 in the ELISA. As shown in Figure 10, 7E1-G2b suppressed the IgG response to SRBCs at doses where the 7E1-G1 did not.

2. 7E1-G2b Dose Response in T-dependent Antigen Mouse Model

7E1-G2b was examined in a T cell dependent primary immune response model using SRBC as the antigen. 7E1-G2b was tested at various doses to determine the lowest effective dose. BALB/c mice (n=5) were injected IV with 1 x 10^8 SRBCs

and treated with a single injection of 7E1-G2b at the indicated doses or MR1 (anti-murine gp39) or PBS administered at the same time as the antigen on day 0. Shown in Figure 11 is the IgG anti-SRBC response on days 7, 16 and 28. Values reported are the ELISA absorbance value at a serum dilution of 1/50. Error bars indicate standard deviation.

As shown in Figure 11, a single treatment with 7E1-G2b at 25 μg/mouse (1.25 mg/kg) suppressed the IgG immune response by 87% on Day 16 and complete suppression was obtained with 50 or 100 μg doses at Day 16. At Day 28, 50 μg/mouse suppressed the IgG response by 89%, and 100 μg/mouse suppressed completely. Note that MR1 was used as a positive control for immunosuppression at a suboptimal dose of 100 μg/mouse.

3. 7E1-G2b in Preventative Collagen-Induced Arthritis (CIA) Mouse Model

A standard experimental murine model for rheumatoid arthritis, the collagen-induced arthritis model (CIA), was used to determine the effect of 7E1-G2b on prevention of arthritis. DBA/1J male mice (6-8 weeks) were injected with 200 ug of chicken collagen type II (CII) in complete Freund's adjuvant intradermally on day 0. Treatment with 7E1-G2b at 250 μg/dose was administered IP every 4 days starting on day 7. The control group was treated with PBS on the same dosing schedule. All mice were boosted with CII in incomplete Freund's adjuvant on day 21. Mice were observed daily for paw swelling and subjectively scored on a scale of 0-3 with 3 equal to maximum swelling and erythema. Paws were also measured with calipers daily. The clinical score reported was derived by summation of the score of each paw at the time of sacrifice and dividing by the total number of animal in each group. The values reported are the median range of the groups.

Arthritis development, and hence joint inflammation in the mice, was completely inhibited by therapy with 7E1-G2b as shown in Table 4 below. Mice treated with 7E1-G2b were completely free of disease through 90 days.

Table 4. Treatment of Collagen-Induced Arthritis

Tx Group	Arthritis Incidence	Median (Range) Day of onset	Median (Range) Clinical score	Median (Range) Paw measure
7E1-G1	0/5	0	0	0.075

7E1-G2b	0/5	0	0	0.075
PBS control	4/4	30 (27 - 32)	3.5 (3 - 4)	0.114 (0.110-0.117)

As demonstrated above, the antibodies of the present invention are potent immunomodulators, with therapeutic uses against a variety of disease.

The present invention encompasses chimeric and humanized antibodies as described above with additional conservative amino acid substitutions which have substantially no effect on CD40 binding. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

In one aspect, the present invention is directed to producing the chimeric and/or humanized antibodies as described above by expressing recombinant DNA segments encoding the murine light variable chain and heavy variable chain (or portions thereof), attached to DNA segments encoding the human constant regions. Exemplary DNA sequences designed in accordance with the present invention code for the polypeptide chains comprising all or a portion of the light chain variable region as shown in SEQ ID NO:1 or its deposited ATCC clone, and/or all or a portion of the heavy chain variable region as shown in SEQ ID NO:2 or its deposited ATCC clone.

Also encompassed within the present invention are the disclosed heavy and light chain variable regions and active or functional parts thereof. The immunologically competent or functional form of the protein or part thereof is also referred to herein as a "light/heavy chain variable region or biologically active portion thereof". In the present case, a biologically active portion thereof comprises a portion of said light or heavy chain which, when incorporated into an antibody, still permits the antibody to bind to human CD40.

Specifically encompassed within the present invention are nucleic acid sequences encoding the variable heavy chain and the variable light chain of an antibody of the present invention. For example, nucleotides 1057 through 1422 (SEQ ID NO:5) of Figure 13 provide a preferred nucleic acid sequence encoding a variable

heavy chain of an antibody of the present invention; nucleotides 1065 through 1388 (SEQ ID NO:6) of Figure 14 provide a preferred nucleic acid sequence encoding a variable light chain of an antibody of the present invention. SEQ ID NO:7 and SEQ ID NO:11 show preferred nucleic acid sequences encoding variable light chains of humanized antibodies of the present invention; SEQ ID NO:9 shows a preferred nucleic acid sequence encoding a variable heavy chain of a humanized antibody of the present invention. Plasmids comprising the polynucleotides shown in SEQ ID NO:7, SEQ ID NO:9 and SEQ ID NO:11 have been deposited with the ATCC.

Chimeric and/or humanized antibodies that bind to human CD40 and that comprise polypeptides that are substantially homologous to, or that show substantial sequence identity to, the variable light and heavy chain sequences disclosed herein are also contemplated by the present invention. For example, chimeric antibodies comprising a light chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the light chain region as shown in SEQ ID NO:4 are included within the scope of the present invention. More particularly, chimeric antibodies comprising a variable light chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the variable light chain region as shown in SEQ ID NO:1 are also included within the scope of the present invention. Also within the scope of the present invention are humanized antibodies comprising a light chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the light chain region as shown in SEQ ID NO:8 and/or SEQ ID NO:12.

Additionally, chimeric antibodies comprising a heavy chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the heavy chain region as

shown in SEQ ID NO:3 are included within the scope of the present invention. More particularly, chimeric antibodies comprising a variable heavy chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the variable heavy chain region as shown in SEQ ID NO:2 are also included within the scope of the present invention. Additionally, humanized antibodies comprising a variable heavy chain region that exhibits at least about 85% sequence identity, more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, and most preferably at least about 98% sequence identity with the variable heavy chain region as shown in SEQ ID NO:10 are also included within the scope of the present invention.

The DNA segments typically further comprise an expression control DNA sequence operably linked to the chimeric or humanized antibody coding sequences, including naturally-associated or heterologous promoter regions. Preferably, the expression control sequences will be eukaryotic promoter systems in vectors capable of transforming or transfecting eukaryotic host cells, but control sequences for prokaryotic hosts may also be used. Once the vector has been incorporated into an appropriate host, the host is maintained under conditions suitable for high level expression of the nucleotide sequences and, as desired, the collection and purification of the variable light chain, heavy chain, light/heavy chain dimers or intact antibody, binding fragments or other immunoglobulin form may follow. (See, Beychok, S., "Cells of Immunoglobulin Synthesis", Academic Press, N.Y. (1979)). Single chain antibodies may also be produced by joining nucleic acid sequences encoding the VL and VH regions disclosed herein with DNA encoding a polypeptide linker.

Prokaryotic hosts, such as E. coli, and other microbes, such as yeast, may be used to express an antibody of the present invention. In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the antibodies of the present invention. Eukaryotic cells may be preferred, because a number of suitable host cell lines capable of secreting intact immunoglobulins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa

cells, myeloma cell lines, and hybridomas. Expression vectors for these cells can include expression control sequences, such as a promoter or enhancer, and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences, all known in the art.

The vectors containing the DNA segments of interest (e.g., the heavy and/or light chain encoding sequences and expression control sequences) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See, e.g., Maniatis, et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Press (1982)).

Once expressed, the whole antibodies, their dimers, individual light and heavy chains, or other immunoglobulin forms of the present invention, can be purified according to standard procedures in the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the like. Substantially pure immunoglobulins of at least 90 to 95% homogeneity are preferred, and 98 to 99% or more homogeneity are most preferred, for pharmaceutical uses.

The antibodies of the present invention will typically find use in treating antibody mediated and/or T cell mediated disorders. Typical disease states suitable for treatment include graft versus host disease and transplant rejection, and autoimmune diseases such as Type I diabetes, psoriasis, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, and myesthenia gravis.

The antibodies and pharmaceutical compositions of the present invention are particularly useful for parenteral administration, i.e., subcutaneously, intramuscularly or intravenously. The pharmaceutical compositions for parenteral administration will commonly comprise a solution of the antibody dissolved in an acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers can be used, all well known in the art, e.g., water, buffered water, saline, glycine and the like. These solutions are sterile and generally free of particulate matter. These pharmaceutical compositions may be sterilized by conventional well known sterilization techniques. The compositions may contain pharmaceutically acceptable auxiliary substances as

required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example, sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate, human albumin, etc.

The compositions containing antibodies of the present invention can be administered for prophylactic and/or therapeutic treatments. In therapeutic application, compositions are administered to a patient already suffering from a disease, in an amount sufficient to cure or at least partially arrest the disease and its complications. An amount adequate to accomplish this is defined as a "therapeutically effective dose". Amounts effective for this use will depend upon the severity of the disease state and the general state of the patient's own immune system, and can be determined by one skilled in the art.

In prophylactic applications, compositions containing antibodies of the present invention are administered to a patient not already in the disease state to enhance the patient's resistance (suppress an immune response). Such an amount is defined to be a "prophylactically effective dose". In this use, the precise amounts again depend upon the patient's state of health and general level of immunity. A preferred prophylactic use is for the prevention of transplant rejection, e.g., kidney transplant rejection.

Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the appended claims.

We Claim:

1. A light chain variable region comprising all or a biologically active portion of an amino acid sequence as shown in SEQ ID NO:1 (Figure 4a).
2. A heavy chain variable region comprising all or a biologically active portion of an amino acid sequence as shown in SEQ ID NO:2 (Figure 4b).
3. A chimeric antibody which binds to human CD40 comprising a light chain and a heavy chain, said light chain comprising the light chain variable region of claim1.
4. A chimeric antibody which binds to human CD40 comprising a light chain and a heavy chain, said heavy chain comprising the heavy chain variable region of claim 2.
5. The chimeric antibody of claim 3 wherein said heavy chain comprises the heavy chain variable region of claim 2.
6. A chimeric antibody which binds to human CD40, comprising a light chain and a heavy chain, said light chain comprising all or a biologically active portion of an amino acid sequence as shown in SEQ ID NO:4 and said heavy chain comprising all or a biologically active portion of an amino acid sequence as shown in SEQ ID NO:3.
7. A nucleic acid molecule comprising a nucleotide sequence encoding the light chain variable region of claim 1.
8. A nucleic acid molecule comprising a nucleotide sequence encoding the heavy chain variable region of claim 2.
9. An expression vector comprising a nucleic acid sequence of claim 7.
10. An expression vector comprising a nucleic acid sequence of claim 8.
11. A humanized antibody comprising a portion of the light chain variable region of claim 1.
12. A humanized antibody comprising a portion of the heavy chain variable region of claim 2.
13. A pharmaceutical composition comprising the chimeric antibody of claim 5.
14. A pharmaceutical composition comprising the chimeric antibody of claim 6.

15. A chimeric antibody which binds to human CD40 comprising a light chain variable region and a heavy chain variable region, wherein said light chain variable region comprises an amino acid sequence having at least 90% sequence identity to the light chain variable region of claim 1.

16. A chimeric antibody which binds to human CD40 comprising a light chain variable region and a heavy chain variable region, wherein said heavy chain variable region comprises an amino acid sequence having at least 90% sequence identity to the heavy chain variable region of claim 2.

17. A method of treating a patient suffering from a T cell mediated disorder, said method comprising administering to said patient a therapeutically effective dose of a pharmaceutical composition of claim 14.

18. The nucleic acid molecule of claim 7 comprising the nucleotide sequence as shown in SEQ ID NO:6.

19. The nucleic acid molecule of claim 8 comprising the nucleotide sequence as shown in SEQ ID NO:5.

20. The chimeric antibody of claim 6 comprising a light chain amino acid sequence as shown in SEQ ID NO:4 and a heavy chain amino acid sequence as shown in SEQ ID NO:3.

21. The humanized antibody of claim 11 comprising a light chain variable region as shown in SEQ ID NO:8.

22. The humanized antibody of claim 11 comprising a heavy chain variable region as shown in SEQ ID NO:10.

23. The humanized antibody of claim 12 comprising a light chain variable region as shown in SEQ ID NO:8.

24. The humanized antibody of claim 12 comprising a heavy chain variable region as shown in SEQ ID NO:10.

25. The humanized antibody of claim 11 comprising a light chain variable region as shown in SEQ ID NO:8 and a heavy chain variable region as shown in SEQ ID NO:10.

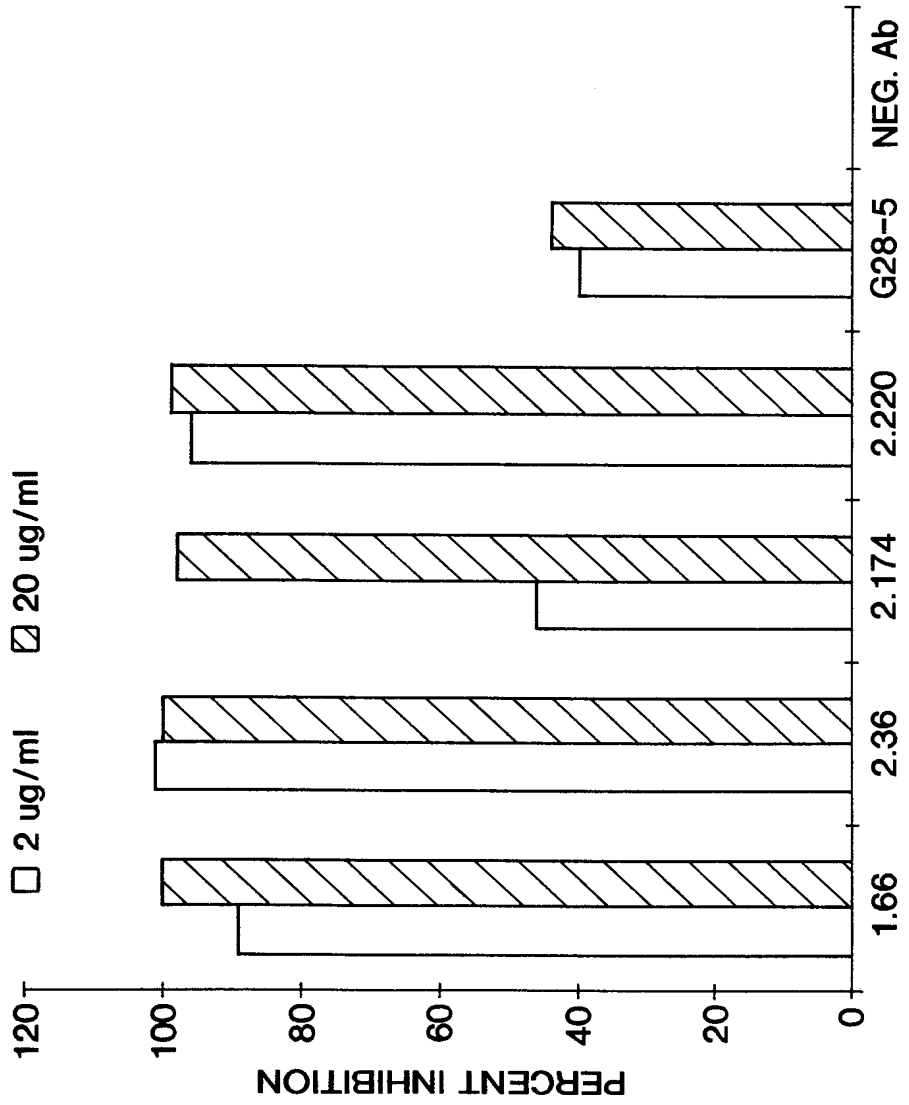
26. The humanized antibody of claim 11 comprising a light chain variable region as shown in SEQ ID NO:12.

27. The humanized antibody of claim 26 comprising a heavy chain variable region as shown in SEQ ID NO:10.

28. The humanized antibody of claim 11 comprising a light chain variable region as shown in SEQ ID NO:12 and a heavy chain variable region as shown in SEQ ID NO:10.

29. A pharmaceutical composition comprising a humanized antibody of claim 25.

30. A pharmaceutical composition comprising a humanized antibody of claim 28.


PERCENT INHIBITION
120
100
80
60
40
20
0
☐ 2 ug/ml
☒ 20 ug/ml
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2.36
2.174
2.220
G28-5
NEG. Ab

FIG. 1

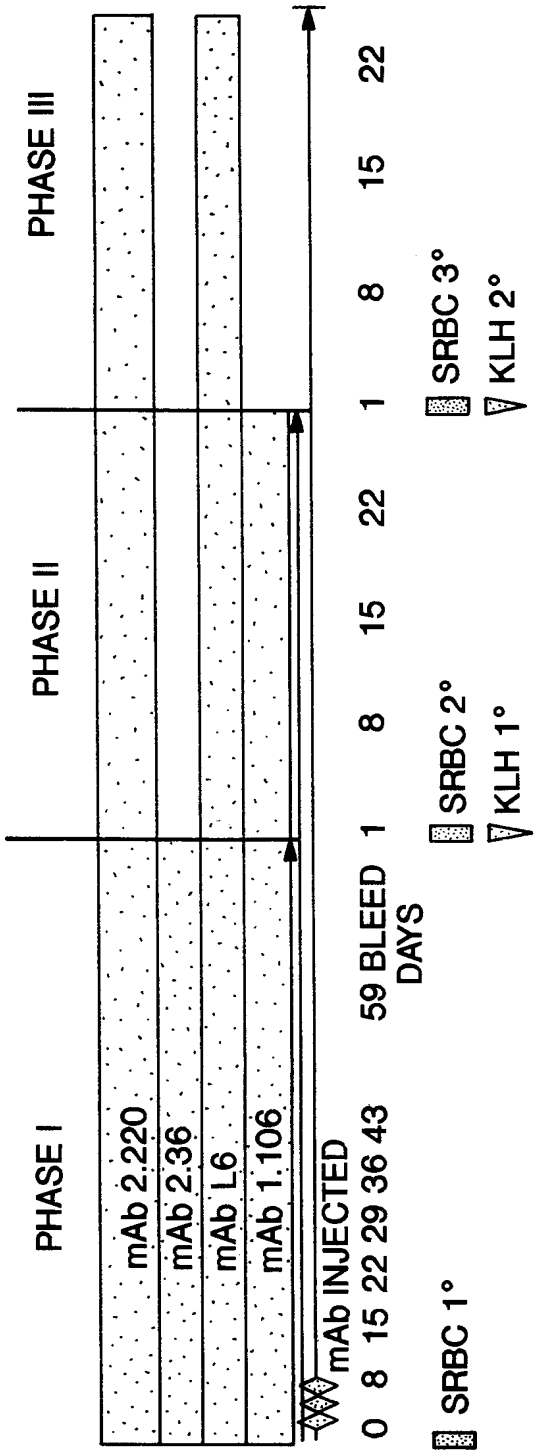
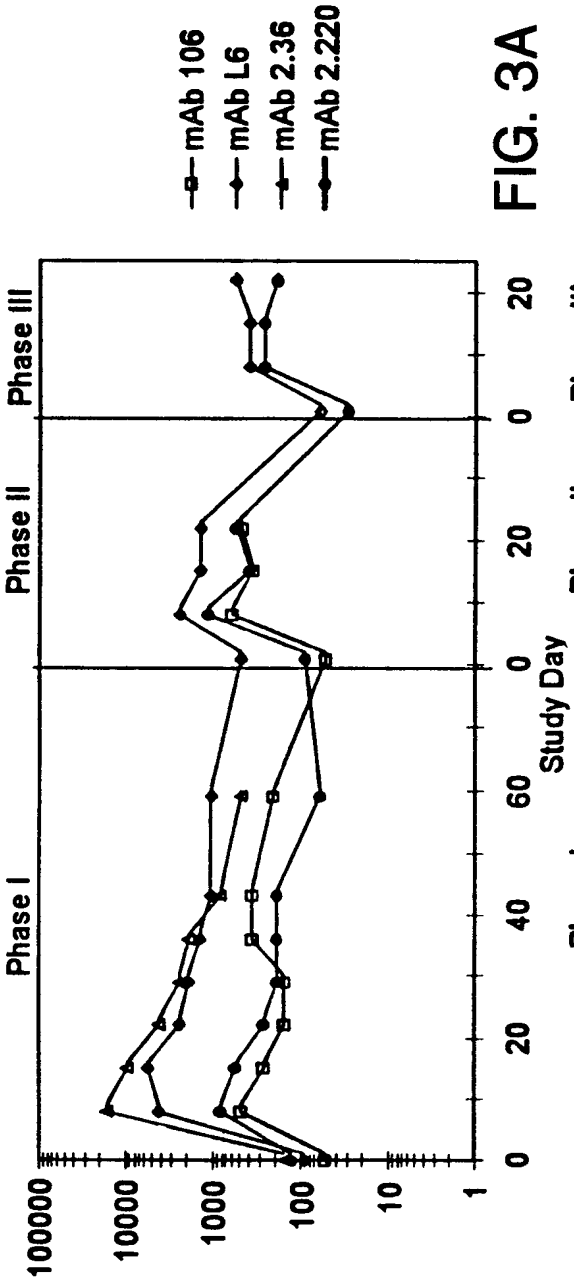

PHASE I
PHASE II
PHASE III
mAb 2.220
mAb 2.36
mAb L6
mAb 1.106
mAb INJECTED
0 8 15 22 29 36 43
59 BLEED DAYS
1 8 15 22 1 8 15 22
SRBC 1°
SRBC 2°
KLH 1°
SRBC 3°
KLH 2°

FIG. 2


mAb 106
mAb L6
mAb 2.36
mAb 2.220
Phase I
Phase II
Phase III
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10000
1000
100
10
1
0
20
40
60
0
20
0
20
Study Day

FIG. 3A

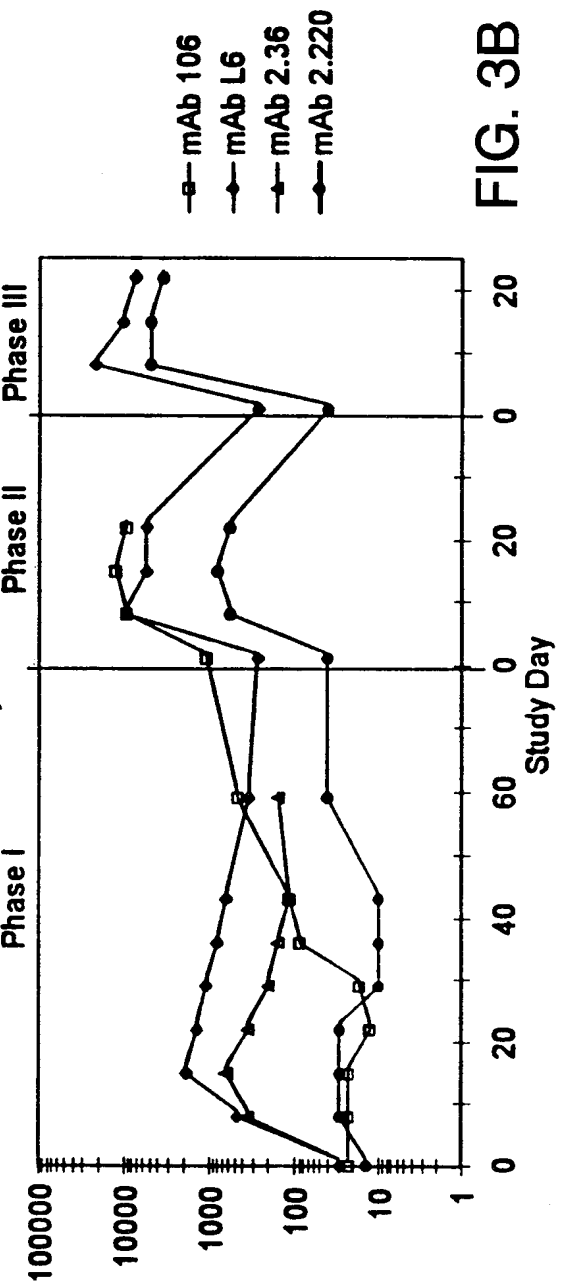
mAb 106
mAb L6
mAb 2.36
mAb 2.220
Phase I
Phase II
Phase III
100000
10000
1000
100
10
1
0
20
40
60
0
20
0
20
Study Day

FIG. 3B

A) Light Chain Variable Region (SEQ ID NO:1).

MEAPAQLLFLLLLWLPDTTGDIVLTQSPATLSVTPGDRVSLSCRASQSISDYLHWYQQKS
HESPRLLIKYASHSISGIPSRFSGSGSGSDFTLSINSVEPEDVGIYYCQHGHSFPWTFGG
GTKLEIKR

B) Heavy Chain Variable Region (SEQ ID NO:2).

MDWTWRILFLVAAATGAHSQIQLVQSGPELKKPGETVRISCKASGYAFTTTGMQWVQEMP
GKGLKWIGWINTHSGVPKYVEDFKGRFAFSLETSANTAYLQISNLKNEDTATYFCVRSGN
GNYDLAYFAYWGQGTLVTVSA

FIG. 4

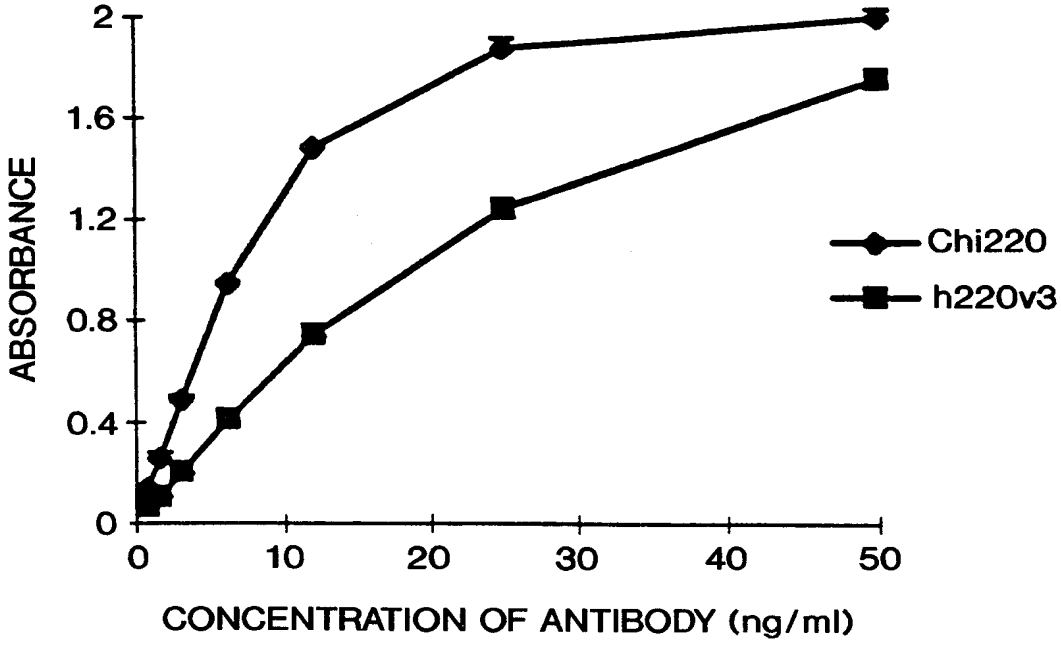
ABSORBANCE
2
1.6
1.2
0.8
0.4
0
0
10
20
30
40
50
CONCENTRATION OF ANTIBODY (ng/ml)
Chi220
h220v3

FIG. 5A

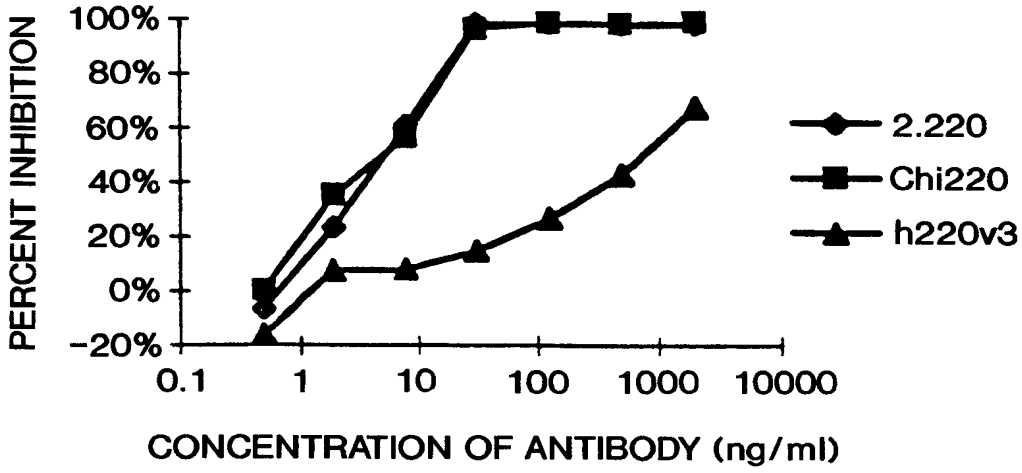
PERCENT INHIBITION
100%
80%
60%
40%
20%
0%
-20%
0.1
1
10
100
1000
10000
CONCENTRATION OF ANTIBODY (ng/ml)
2.220
Chi220
h220v3

FIG. 5B

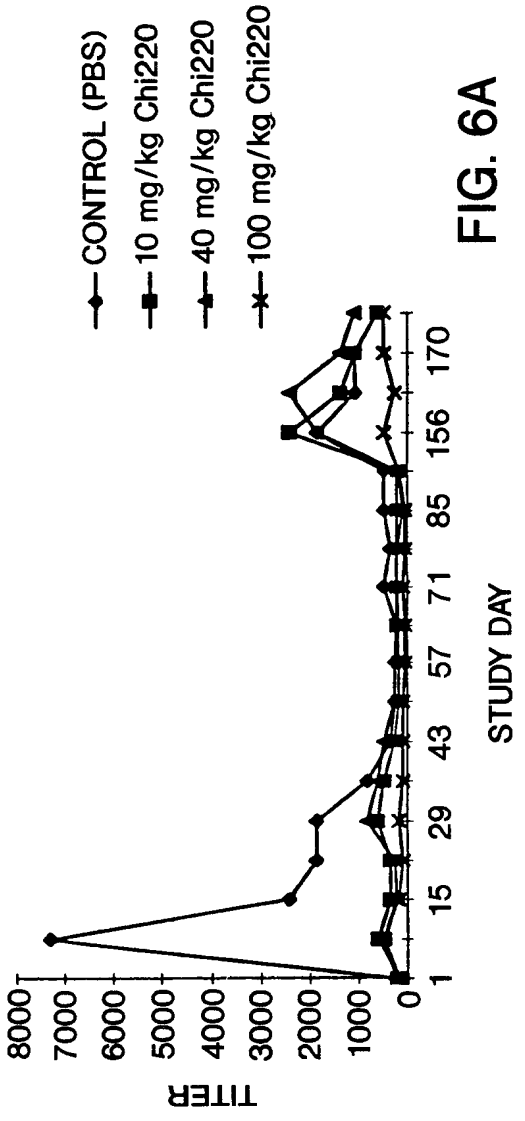
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
TITER
8000
7000
6000
5000
4000
3000
2000
1000
0
1
15
29
43
57
71
85
156
170
STUDY DAY

FIG. 6A

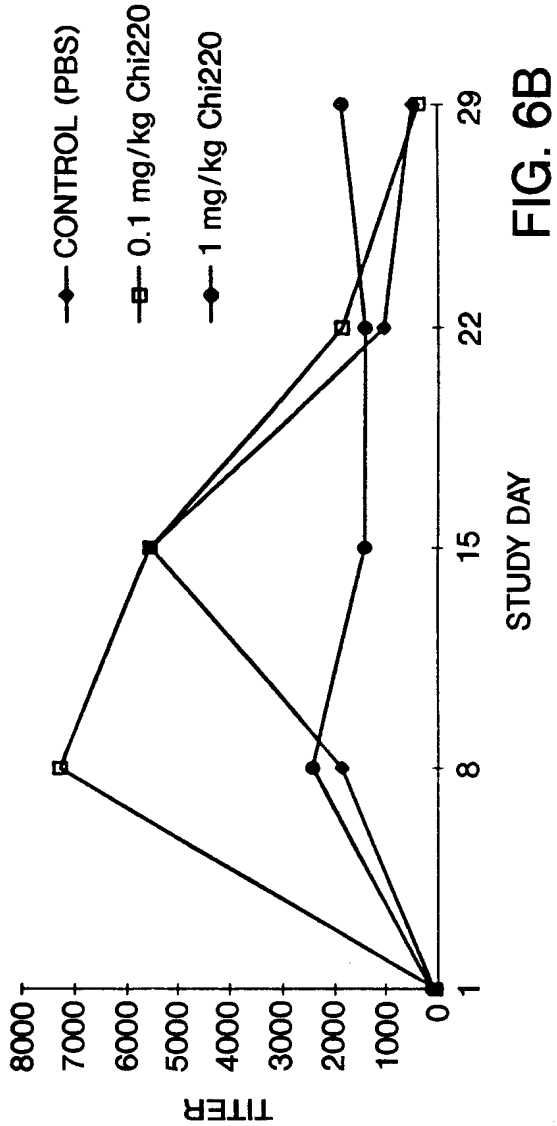
CONTROL (PBS)
0.1 mg/kg Chi220
1 mg/kg Chi220
TITER
8000
7000
6000
5000
4000
3000
2000
1000
0
1
8
15
22
29
STUDY DAY

FIG. 6B

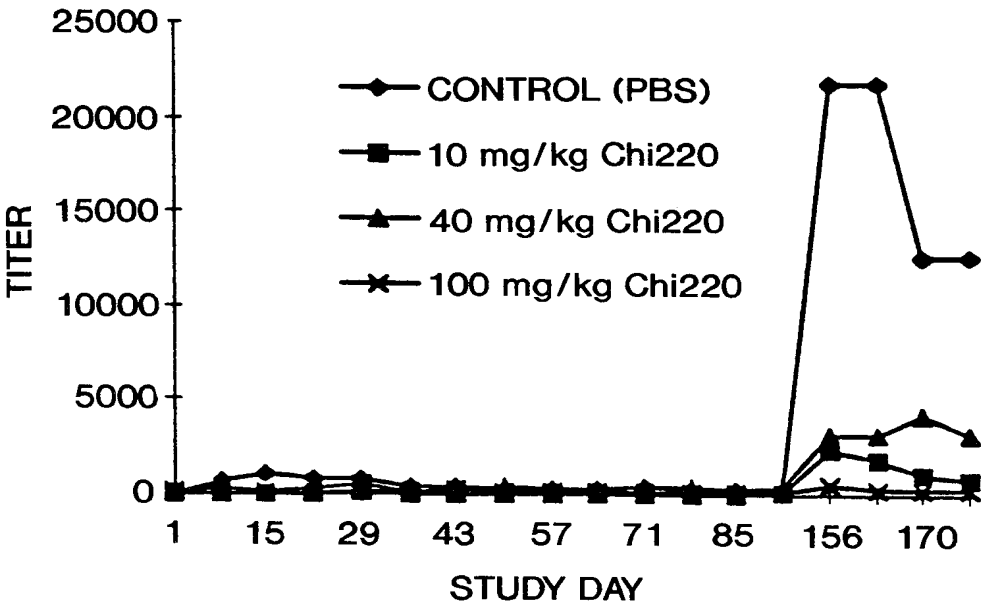
25000
20000
15000
10000
5000
0
TITER
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
1
15
29
43
57
71
85
156
170
STUDY DAY

FIG. 7A

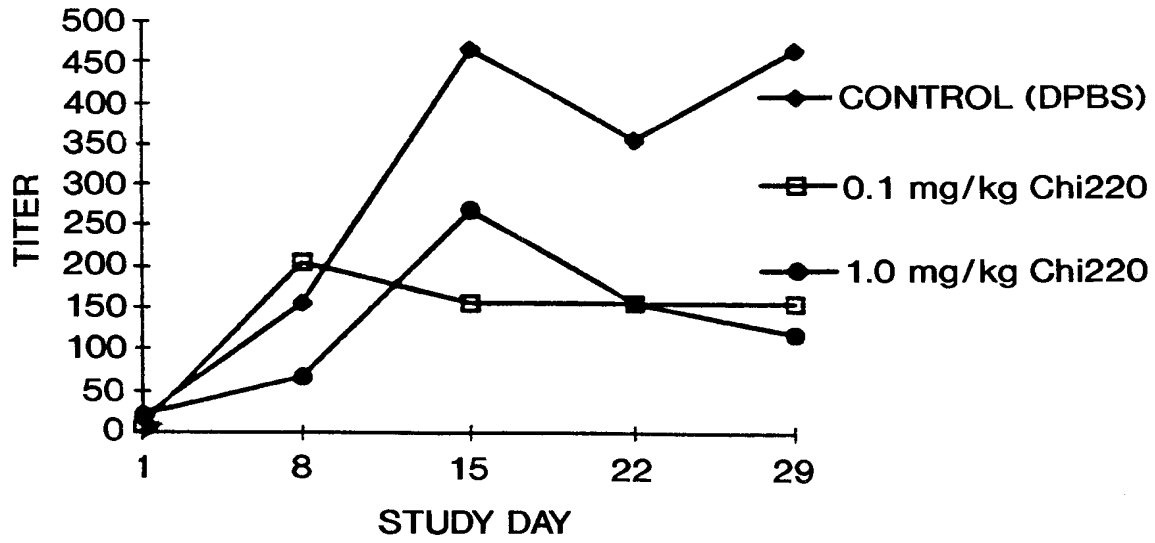
500
450
400
350
300
250
200
150
100
50
0
TITER
CONTROL (DPBS)
0.1 mg/kg Chi220
1.0 mg/kg Chi220
1
8
15
22
29
STUDY DAY

FIG. 7B

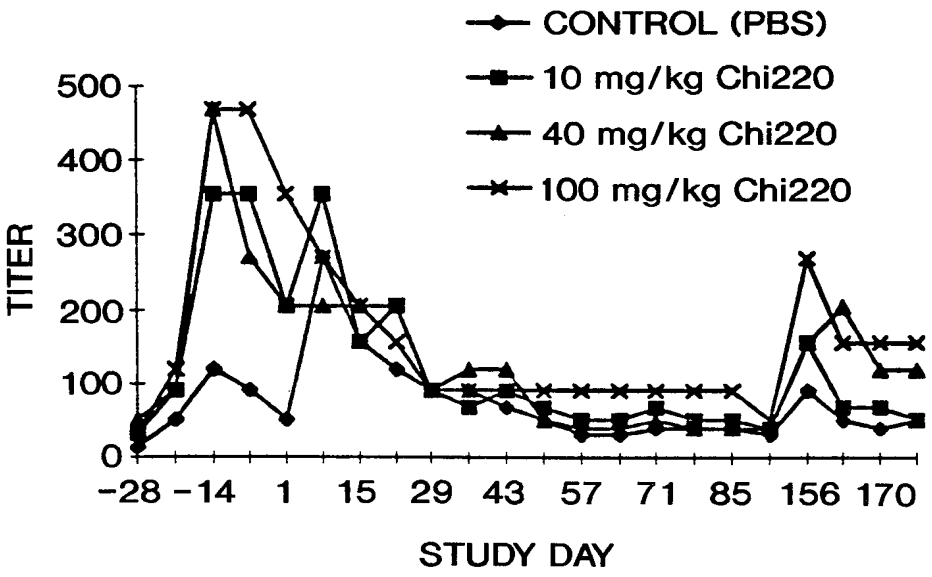
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
TITER
500
400
300
200
100
0
-28 -14 1 15 29 43 57 71 85 156 170
STUDY DAY

FIG. 8A

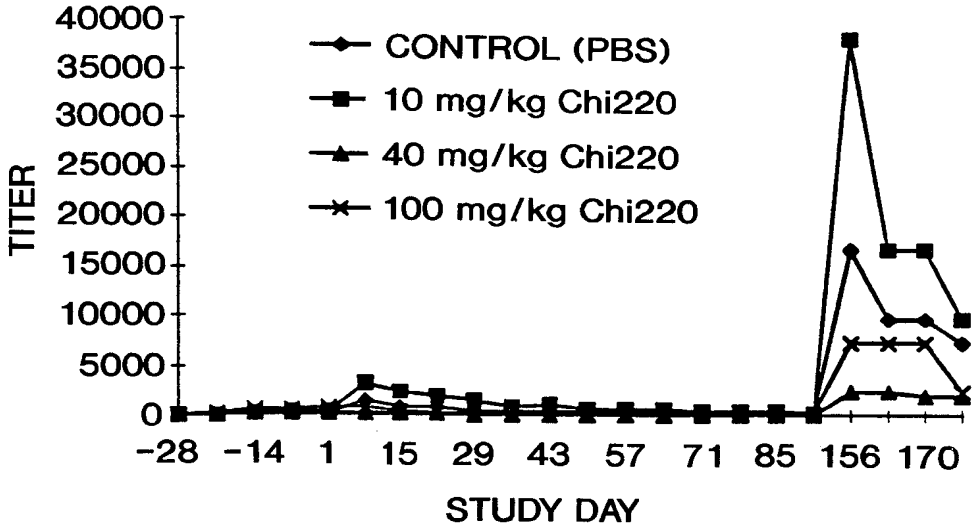
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
TITER
40000
35000
30000
25000
20000
15000
10000
5000
0
-28 -14 1 15 29 43 57 71 85 156 170
STUDY DAY

FIG. 8B

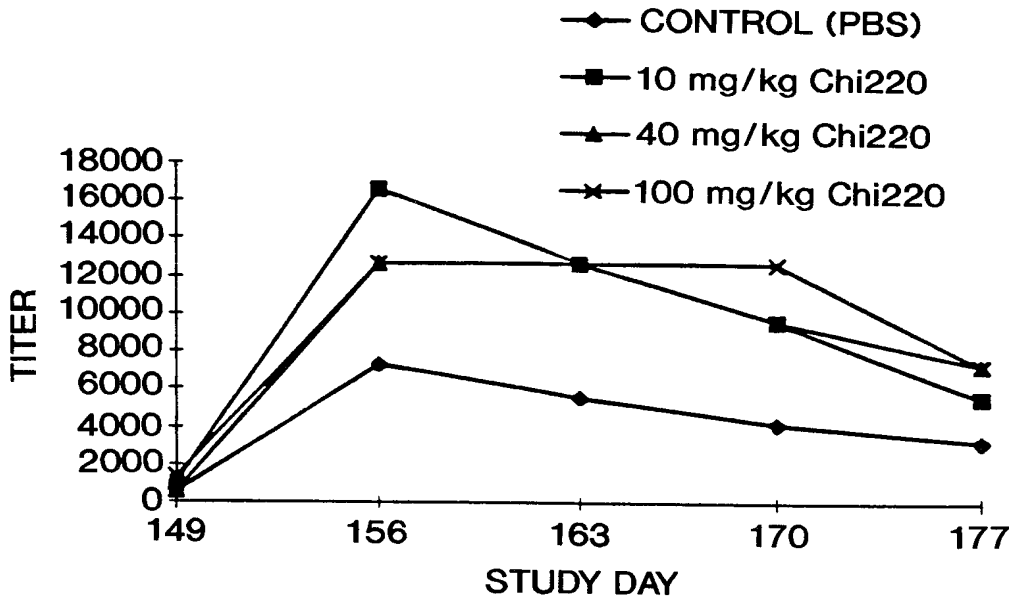
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
TITER
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
149
156
163
170
177
STUDY DAY

FIG. 9A

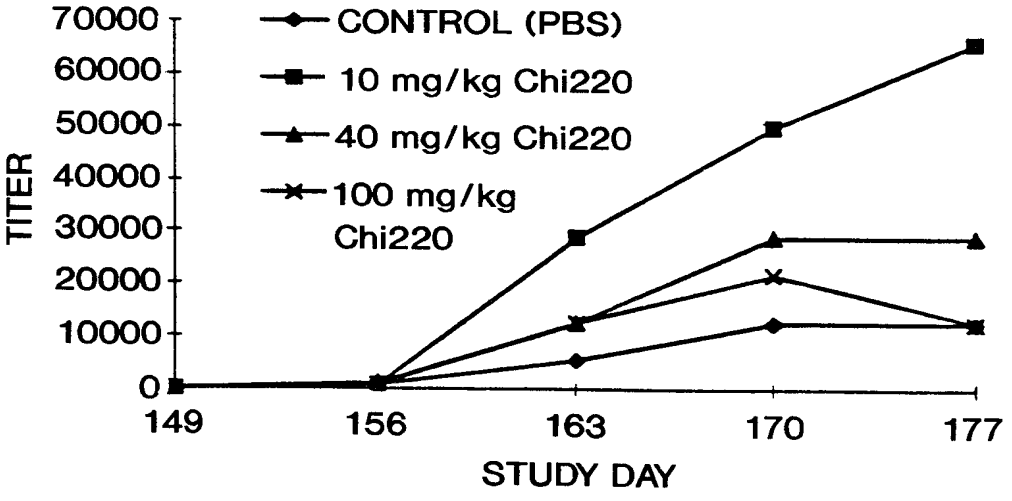
CONTROL (PBS)
10 mg/kg Chi220
40 mg/kg Chi220
100 mg/kg Chi220
TITER
70000
60000
50000
40000
30000
20000
10000
0
149
156
163
170
177
STUDY DAY

FIG. 9B

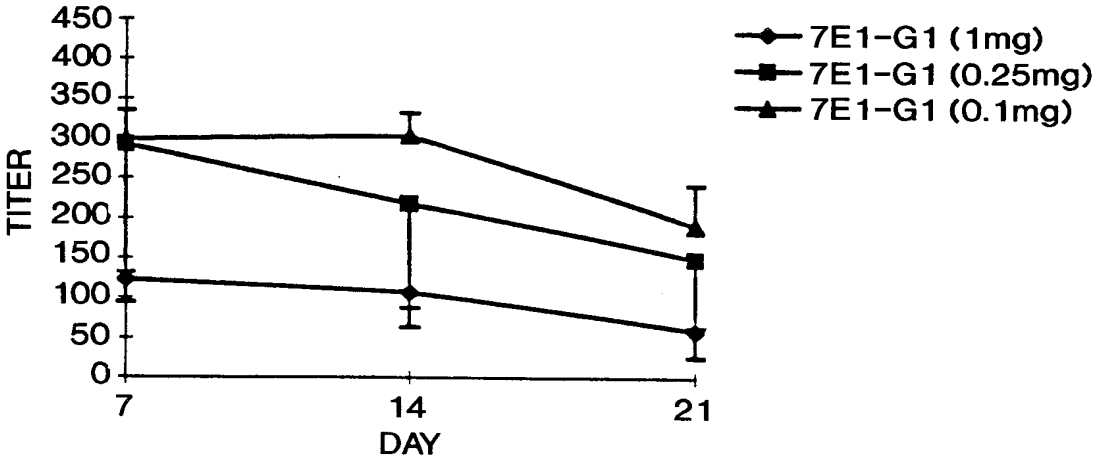

FIG. 10A

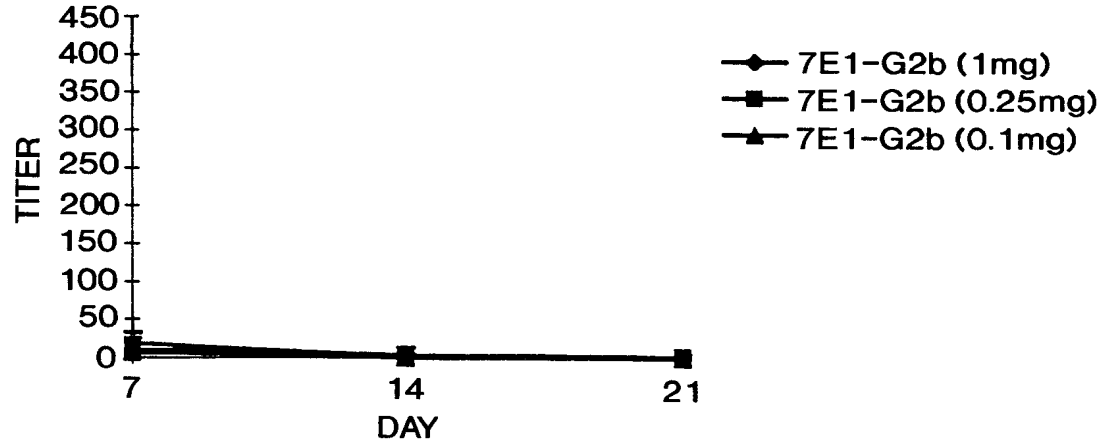

FIG. 10B

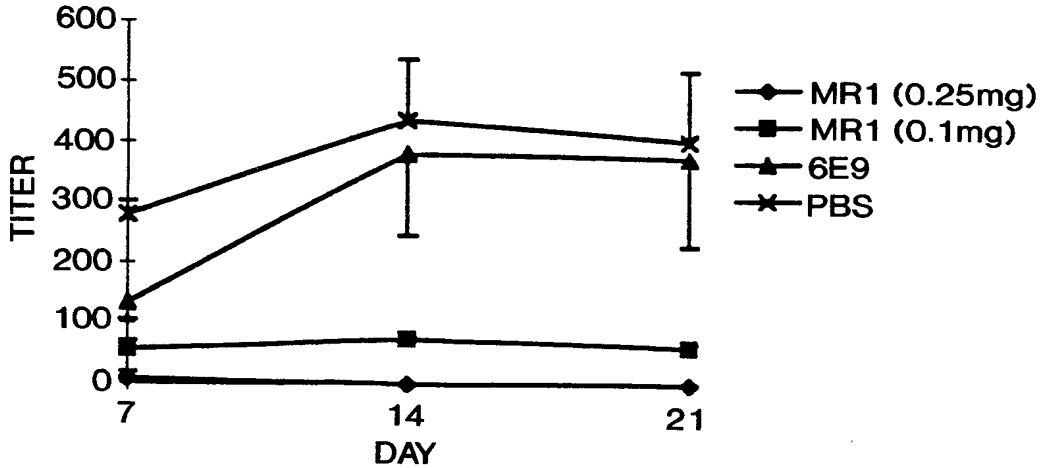

FIG. 10C

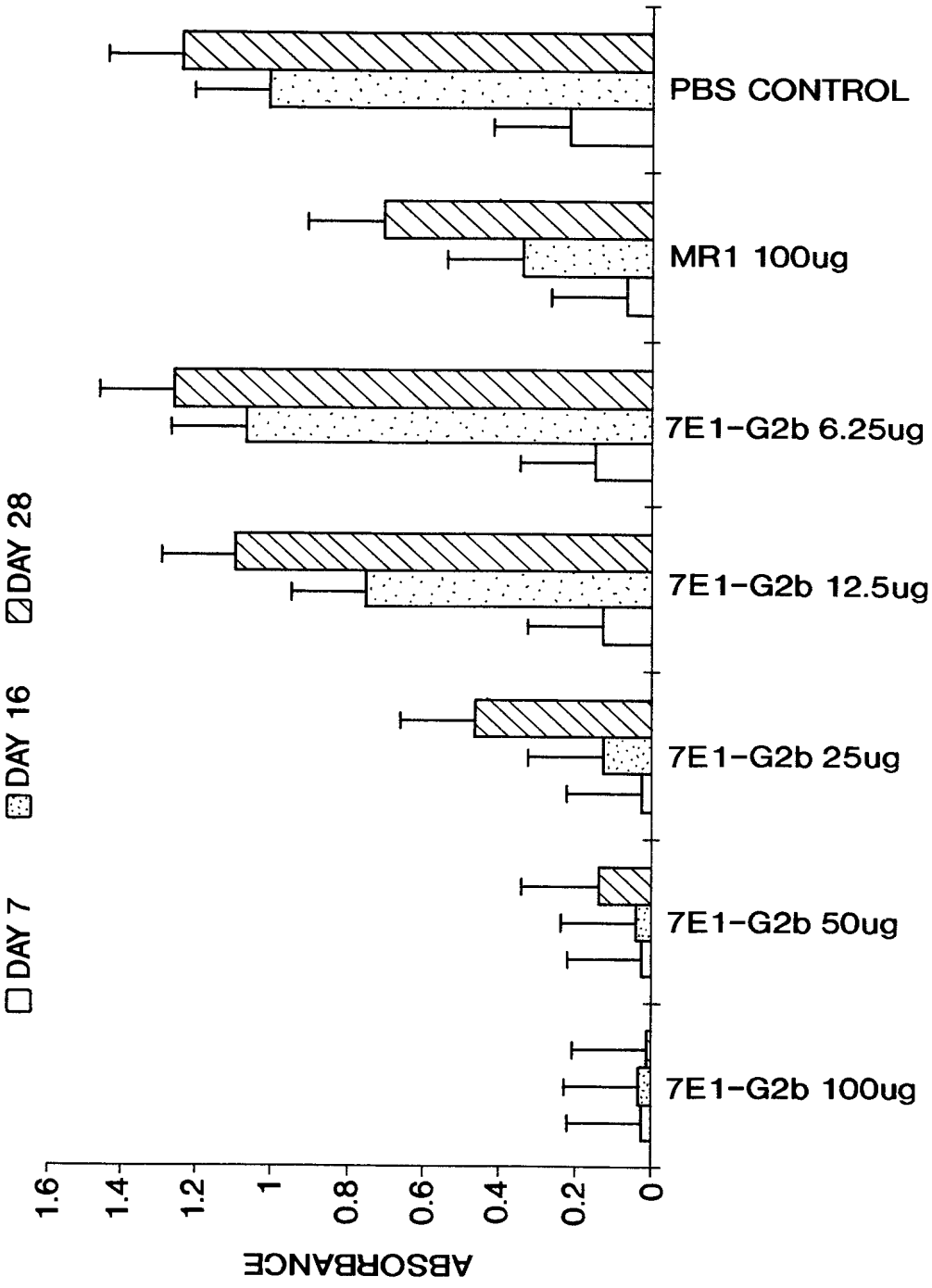
PBS CONTROL
MR1 100ug
7E1-G2b 6.25ug
7E1-G2b 12.5ug
7E1-G2b 25ug
7E1-G2b 50ug
7E1-G2b 100ug
DAY 7
DAY 16
DAY 28
ABSORBANCE
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6

FIG. 11

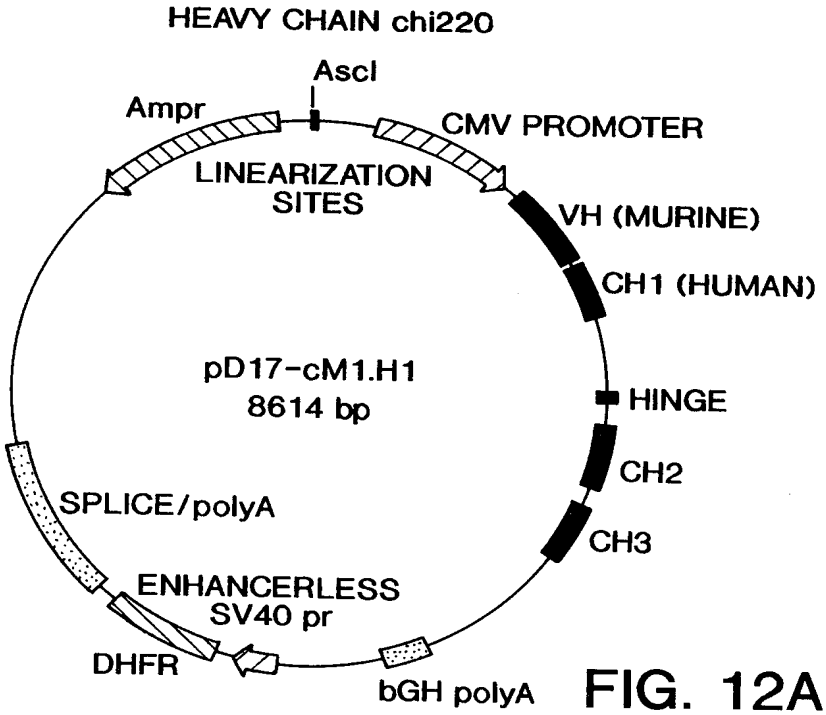

FIG. 12A

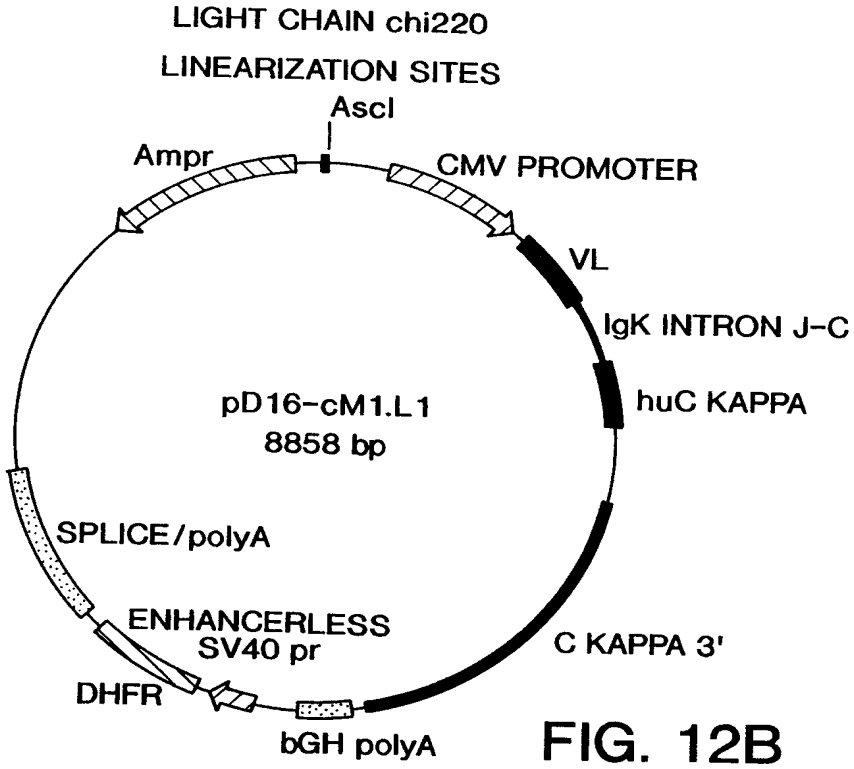

FIG. 12B

```
1    GACGGATCGG GAGATCTGCT AGGTGACCTG AGGCGCGCCG GCTTCGAATA GCCAGAGTAA
61   CCTTTTTTTT TAATTTTATT TTATTTTATT TTTGAGATGG AGTTTGGCGC CGATCTCCCG
121  ATCCCCTATG GTCGACTCTC AGTACAATCT GCTCTGATGC CGCATAGTTA AGCCAGTATC
181  TGCTCCCTGC TTGTGTGTTG GAGGTCGCTG AGTAGTGCGC GAGCAAAATT TAAGCTACAA
241  CAAGGCAAGG CTTGACCGAC AATTGCATGA AGAATCTGCT TAGGGTTAGG CGTTTTGCGC
301  TGCTTCGCGA TGTACGGGCC AGATATACGC GTTGACATTG ATTATTGACT AGTTATTAAT
361  AGTAATCAAT TACGGGGTCA TTAGTTCATA GCCCATATAT GGAGTTCCGC GTTACATAAC
421  TTACGGTAAA TGGCCCGCCT GGCTGACCGC CCAACGACCC CCGCCCATTG ACGTCAATAA
481  TGACGTATGT TCCCATAGTA ACGCCAATAG GGACTTTCCA TTGACGTCAA TGGGTGGACT
541  ATTTACGGTA AACTGCCCAC TTGGCAGTAC ATCAAGTGTA TCATATGCCA AGTACGCCCC
601  CTATTGACGT CAATGACGGT AAATGGCCCG CCTGGCATTA TGCCCAGTAC ATGACCTTAT
661  GGGACTTTCC TACTTGGCAG TACATCTACG TATTAGTCAT CGCTATTACC ATGGTGATGC
721  GGTTTTGGCA GTACATCAAT GGGCGTGGAT AGCGGTTTGA CTCACGGGGA TTTCCAAGTC
781  TCCACCCCAT TGACGTCAAT GGGAGTTTGT TTTGGCACCA AAATCAACGG GACTTTCCAA
841  AATGTCGTAA CAACTCCGCC CCATTGACGC AAATGGGCGG TAGGCGTGTA CGGTGGGAGG
901  TCTATATAAG CAGAGCTCTC TGGCTAACTA GAGAACCCAC TGCTTACTGG CTTATCGAAA
961  TTAATACGAC TCACTATAGG GAGACCCAAG CTTGGTACCA TGGACTGGAC CTGGAGAATC
1021 CTCTTCTTGG TGGCAGCAGC AACAGGTGCC CACTCCCAGA TCCAGTTGGT GCAATCTGGA
1081 CCTGAGCTGA AGAAGCCTGG AGAGACAGTC AGGATCTCCT GCAAGGCTTC TGGGTATGCC
1141 TTCACAACTA CTGGAATGCA GTGGGTGCAA GAGATGCCAG GAAAGGGTTT GAAGTGGATT
1201 GGCTGGATAA ACACCCACTC TGGAGTGCCA AAATATGTAG AAGACTTCAA GGGACGGTTT
1261 GCCTTCTCTT TGGAAACCTC TGCCAACACT GCATATTTAC AGATAAGCAA CCTCAAAAAT
1321 GAGGACACGG CTACGTATTT CTGTGTGAGA TCCGGGAATG GTAACTATGA CCTGGCCTAC
1381 TTTGCTTACT GGGGCCAAGG GACACTGGTC ACTGTCTCTG CAGCTAGCAC CAAGGGCCCA
1441 TCGGTCTTCC CCCTGGCACC CTCCTCCAAG AGCACCTCTG GGGGCACAGC GGCCCTGGGC
1501 TGCCTGGTCA AGGACTACTT CCCCGAACCG GTGACGGTGT CGTGGAACTC AGGCGCCCTG
1561 ACCAGCGGCG TGCACACCTT CCCGGCTGTC CTACAGTCCT CAGGACTCTA CTCCCTCAGC
1621 AGCGTGGTGA CCGTGCCCTC CAGCAGCTTG GGCACCCAGA CCTACATCTG CAACGTGAAT
1681 CACAAGCCCA GCAACACCAA GGTGGACAAG AAAGTTGGTG AGAGGCCAGC ACAGGGAGGG
1741 AGGGTGTCTG CTGGAAGCCA GGCTCAGCGC TCCTGCCTGG ACGCATCCCG GCTATGCAGC
1801 CCCAGTCCAG GGCAGCAAGG CAGGCCCCGT CTGCCTCTTC ACCCGGAGGC CTCTGCCCGC
1861 CCCACTCATG CTCAGGGAGA GGGTCTTCTG GCTTTTTCCC CAGGCTCTGG GCAGGCACAG
1921 GCTAGGTGCC CCTAACCCAG GCCCTGCACA CAAAGGGGCA GGTGCTGGGC TCAGACCTGC
1981 CAAGAGCCAT ATCCGGGAGG ACCCTGCCCC TGACCTAAGC CCACCCCAAA GGCCAAACTC
2041 TCCACTCCCT CAGCTCGGAC ACCTTCTCTC CTCCCAGATT CCAGTAACTC CCAATCTTCT
2101 CTCTGCAGAG CCCAAATCTT GTGACAAAAC TCACACATGC CCACCGTGCC CAGGTAAGCC
2161 AGCCCAGGCC TCGCCCTCCA GCTCAAGGCG GGACAGGTGC CCTAGAGTAG CCTGCATCCA
2221 GGGACAGGCC CCAGCCGGGT GCTGACACGT CCACCTCCAT CTCTTCCTCA GCACCTGAAC
2281 TCCTGGGGGG ACCGTCAGTC TTCCTCTTCC CCCCAAAACC CAAGGACACC CTCATGATCT
2341 CCCGGACCCC TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC CCTGAGGTCA
2401 AGTTCAACTG GTACGTGGAC GGCGTGGAGG TGCATAATGC CAAGACAAAG CCGCGGGAGG
2461 AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC CAGGACTGGC
2521 TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC CCTCCCAGCC CCCATCGAGA
2581 AAACCATCTC CAAAGCCAAA GGTGGGACCC GTGGGGTGCG AGGGCCACAT GGACAGAGGC
2641 CGGCTCGGCC CACCCTCTGC CCTGAGAGTG ACCGCTGTAC CAACCTCTGT CCCTACAGGG
2701 CAGCCCCGAG AACCACAGGT GTACACCCTG CCCCCATCCC GGGATGAGCT GACCAAGAAC
2761 CAGGTCAGCC TGACCTGCCT GGTCAAAGGC TTCTATCCCA GCGACATCGC CGTGGAGTGG
2821 GAGAGCAATG GGCAGCCGGA GAACAACTAC AAGACCACGC CTCCCGTGCT GGACTCCGAC
2881 GGCTCCTTCT TCCTCTACAG CAAGCTCACC GTGGACAAGA GCAGGTGGCA GCAGGGGAAC
2941 GTCTTCTCAT GCTCCGTGAT GCATGAGGCT CTGCACAACC ACTACACGCA GAAGAGCCTC
3001 TCCCTGTCTC CGGGTAAATG AGTGCGACGG CCGGCAAGCC CCCGCTCCCC GGGCTCTCGC
```

FIG. 13A

```
3061 GGTCGCACGA GGATGCTTGG CACGTACCCC CTGTACATAC TTCCCGGGCG CCCAGCATGG
3121 AAATAAAGCA CCCAGCGCTG CCCTGGGCCC CTGCGAGACT GTGATGGTTC TTTCCACGGG
3181 TCAGGCCGAG TCTGAGGCCT GAGTGGCATG AGGGAGGCAG AGCGGGTCCC ACTGTCCCCA
3241 CACTGGCCCA GGCTGTGCAG GTGTGCCTGG GCCCCCTAGG GTGGGGCTCA GCCAGGGGCT
3301 GCCCTCGGCA GGGTGGGGGA TTTGCCAGCG TGGCCCTCCC TCCAGCAGCA CCTGCCCTGG
3361 GCTGGGCCAC GGGAAGCCCT AGGAGCCCCT GGGGACAGAC ACACAGCCCC TGCCTCTGTA
3421 GGAGACTGTC CTGTTCTGTG AGCGCCCCTG TCCTCCCGAC CTCCATGCCC ACTCGGGGGC
3481 ATGCCTAGTC CATGTGCGTA GGGACAGGCC CTCCCTCACC CATCTACCCC CACGGCACTA
3541 ACCCCTGGCT GCCCTGCCCA GCCTCGCACC CGCATGGGGA CACAACCGAC TCCGGGGACA
3601 TGCACTCTCG GGCCCTGTGG AGGGACTGGT GCAGATGCCC ACACACACAC TCAGCCCAGA
3661 CCCGTTCAAC AAACCCCGCA CTGAGGTTGG CCGGCCACAC GGCCACCACA CACACACGTG
3721 CACGCCTCAC ACACGGAGCC TCACCCGGGC GAACTGCACA GCACCCAGAC CAGAGCAAGG
3781 TCCTCGCACA CGTGAACACT CCTCGGACAC AGGCCCCCAC GAGCCCCACG CGGCACCTCA
3841 AGGCCCACGA GCCTCTCGGC AGCTTCTCCA CATGCTGACC TGCTCAGACA AACCCAGCCC
3901 TCCTCTCACA AGGGTGCCCC TGCAGCCGCC ACACACACAC AGGGGATCAC ACACCACGTC
3961 ACGTCCCTGG CCCTGGCCCA CTTCCCAGTG CCGCCCTTCC CTGCAGGACG GATCAGCCTC
4021 GACTGTGCCT TCTAGTTGCC AGCCATCTGT TGTTTGCCCC TCCCCCGTGC CTTCCTTGAC
4081 CCTGGAAGGT GCCACTCCCA CTGTCCTTTC CTAATAAAAT GAGGAAATTG CATCGCATTG
4141 TCTGAGTAGG TGTCATTCTA TTCTGGGGGG TGGGGTGGGG CAGGACAGCA AGGGGGAGGA
4201 TTGGGAAGAC AATAGCAGGC ATGCTGGGGA TGCGGTGGGC TCTATGGCTT CTGAGGCGGA
4261 AAGAACCAGC TGGGGCTCTA GGGGGTATCC CCACGCGCCC TGTAGCGGCG CATTAAGCGC
4321 GGCGGGTGTG GTGGTTACGC GCAGCGTGAC CGCTACACTT GCCAGCGCCC TAGCGCCCGC
4381 TCCTTTCGCT TTCTTCCCTT CCTTTCTCGC CACGTTCGCC GGGCCTCTCA AAAAAGGGAA
4441 AAAAAGCATG CATCTCAATT AGTCAGCAAC CATAGTCCCG CCCCTAACTC CGCCCATCCC
4501 GCCCCTAACT CCGCCCAGTT CCGCCCATTC TCCGCCCCAT GGCTGACTAA TTTTTTTTAT
4561 TTATGCAGAG GCCGAGGCCG CCTCGGCCTC TGAGCTATTC CAGAAGTAGT GAGGAGGCTT
4621 TTTTGGAGGC CTAGGCTTTT GCAAAAAGCT TGGACAGCTC AGGGCTGCGA TTTCGCGCCA
4681 AACTTGACGG CAATCCTAGC GTGAAGGCTG GTAGGATTTT ATCCCCGCTG CCATCATGGT
4741 TCGACCATTG AACTGCATCG TCGCCGTGTC CCAAAATATG GGGATTGGCA AGAACGGAGA
4801 CCTACCCTGG CCTCCGCTCA GGAACGAGTT CAAGTACTTC CAAAGAATGA CCACAACCTC
4861 TTCAGTGGAA GGTAAACAGA ATCTGGTGAT TATGGGTAGG AAAACCTGGT TCTCCATTCC
4921 TGAGAAGAAT CGACCTTTAA AGGACAGAAT TAATATAGTT CTCAGTAGAG AACTCAAAGA
4981 ACCACCACGA GGAGCTCATT TTCTTGCCAA AAGTTTGGAT GATGCCTTAA GACTTATTGA
5041 ACAACCGGAA TTGGCAAGTA AAGTAGACAT GGTTTGGATA GTCGGAGGCA GTTCTGTTTA
5101 CCAGGAAGCC ATGAATCAAC CAGGCCACCT TAGACTCTTT GTGACAAGGA TCATGCAGGA
5161 ATTTGAAAGT GACACGTTTT TCCCAGAAAT TGATTTGGGG AAATATAAAC TTCTCCCAGA
5221 ATACCCAGGC GTCCTCTCTG AGGTCCAGGA GGAAAAAGGC ATCAAGTATA AGTTTGAAGT
5281 CTACGAGAAG AAAGACTAAC AGGAAGATGC TTTCAAGTTC TCTGCTCCCC TCCTAAAGCT
5341 ATGCATTTTT ATAAGACCAT GGGACTTTTG CTGGCTTTAG ATCTCTTTGT GAAGGAACCT
5401 TACTTCTGTG GTGTGACATA ATTGGACAAA CTACCTACAG AGATTTAAAG CTCTAAGGTA
5461 AATATAAAAT TTTTAAGTGT ATAATGTGTT AAACTACTGA TTCTAATTGT TTGTGTATTT
5521 TAGATTCCAA CCTATGGAAC TGATGAATGG GAGCAGTGGT GGAATGCCTT TAATGAGGAA
5581 AACCTGTTTT GCTCAGAAGA AATGCCATCT AGTGATGATG AGGCTACTGC TGACTCTCAA
5641 CATTCTACTC CTCCAAAAAA GAAGAGAAAG GTAGAAGACC CCAAGGACTT TCCTTCAGAA
5701 TTGCTAAGTT TTTTGAGTCA TGCTGTGTTT AGTAATAGAA CTCTTGCTTG CTTTGCTATT
5761 TACACCACAA AGGAAAAAGC TGCACTGCTA TACAAGAAAA TTATGGAAAA ATATTCTGTA
5821 ACCTTTATAA GTAGGCATAA CAGTTATAAT CATAACATAC TGTTTTTTCT TACTCCACAC
5881 AGGCATAGAG TGTCTGCTAT TAATAACTAT GCTCAAAAAT TGTGTACCTT TAGCTTTTTA
5941 ATTTGTAAAG GGGTTAATAA GGAATATTTG ATGTATAGTG CCTTGACTAG AGATCATAAT
6001 CAGCCATACC ACATTTGTAG AGGTTTTACT TGCTTTAAAA AACCTCCCAC ACCTCCCCCT
6061 GAACCTGAAA CATAAAATGA ATGCAATTGT TGTTGTTAAC TTGTTTATTG CAGCTTATAA
```

FIG. 13B

```
6121 TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT AAAGCATTTT TTTCACTGCA
6181 TTCTAGTTGT GGTTTGTCCA AACTCATCAA TGTATCTTAT CATGTCTGGA TCGGCTGGAT
6241 GATCCTCCAG CGCGGGGATC TCATGCTGGA GTTCTTCGCC CACCCCAACT TGTTTATTGC
6301 AGCTTATAAT GGTTACAAAT AAAGCAATAG CATCACAAAT TTCACAAATA AAGCATTTTT
6361 TTCACTGCAT TCTAGTTGTG GTTTGTCCAA ACTCATCAAT GTATCTTATC ATGTCTGTAT
6421 ACCGTCGACC TCTAGCTAGA GCTTGGCGTA ATCATGGTCA TAGCTGTTTC CTGTGTGAAA
6481 TTGTTATCCG CTCACAATTC CACACAACAT ACGAGCCGGA AGCATAAAGT GTAAAGCCTG
6541 GGGTGCCTAA TGAGTGAGCT AACTCACATT AATTGCGTTG CGCTCACTGC CCGCTTTCCA
6601 GTCGGGAAAC CTGTCGTGCC AGCTGCATTA ATGAATCGGC CAACGCGCGG GGAGAGGCGG
6661 TTTGCGTATT GGGCGCTCTT CCGCTTCCTC GCTCACTGAC TCGCTGCGCT CGGTCGTTCG
6721 GCTGCGGCGA GCGGTATCAG CTCACTCAAA GGCGGTAATA CGGTTATCCA CAGAATCAGG
6781 GGATAACGCA GGAAAGAACA TGTGAGCAAA AGGCCAGCAA AAGGCCAGGA ACCGTAAAAA
6841 GGCCGCGTTG CTGGCGTTTT TCCATAGGCT CCGCCCCCCT GACGAGCATC ACAAAAATCG
6901 ACGCTCAAGT CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC
6961 TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT ACCTGTCCGC
7021 CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCAATGCTCA CGCTGTAGGT ATCTCAGTTC
7081 GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA CCCCCCGTTC AGCCCGACCG
7141 CTGCGCCTTA TCCGGTAACT ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC
7201 ACTGGCAGCA GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA
7261 GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGG ACAGTATTTG GTATCTGCGC
7321 TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC TCTTGATCCG GCAAACAAAC
7381 CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG ATTACGCGCA GAAAAAAAGG
7441 ATCTCAAGAA GATCCTTTGA TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC
7501 ACGTTAAGGG ATTTTGGTCA TGAGATTATC AAAAAGGATC TTCACCTAGA TCCTTTTAAA
7561 TTAAAAATGA AGTTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTTA
7621 CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT CATCCATAGT
7681 TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG GGCTTACCAT CTGGCCCCAG
7741 TGCTGCAATG ATACCGCGAG ACCCACGCTC ACCGGCTCCA GATTTATCAG CAATAAACCA
7801 GCCAGCCGGA AGGGCCGAGC GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC
7861 TATTAATTGT TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT
7921 TGTTGCCATT GCTACAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG CTTCATTCAG
7981 CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC ATGTTGTGCA AAAAAGCGGT
8041 TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG AAGTAAGTTG GCCGCAGTGT TATCACTCAT
8101 GGTTATGGCA GCACTGCATA ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT
8161 GACTGGTGAG TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC
8221 TTGCCCGGCG TCAATACGGG ATAATACCGC GCCACATAGC AGAACTTTAA AAGTGCTCAT
8281 CATTGGAAAA CGTTCTTCGG GGCGAAAACT CTCAAGGATC TTACCGCTGT TGAGATCCAG
8341 TTCGATGTAA CCCACTCGTG CACCCAACTG ATCTTCAGCA TCTTTTACTT TCACCAGCGT
8401 TTCTGGGTGA GCAAAAACAG GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG
8461 GAAATGTTGA ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA
8521 TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA TAGGGGTTCC
8581 GCGCACATTT CCCCGAAAAG TGCCACCTGA CGTC
```

FIG. 13C

```
1     GACGGATCGG GAGATCTGCT AGCCCGGGTG ACCTGAGGCG CGCCGGCTTC GAATAGCCAG
61    AGTAACCTTT TTTTTTAATT TTATTTTATT TTATTTTTGA GATGGAGTTT GGCGCCGATC
121   TCCCGATCCC CTATGGTCGA CTCTCAGTAC AATCTGCTCT GATGCCGCAT AGTTAAGCCA
181   GTATCTGCTC CCTGCTTGTG TGTTGGAGGT CGCTGAGTAG TGCGCGAGCA AAATTTAAGC
241   TACAACAAGG CAAGGCTTGA CCGACAATTG CATGAAGAAT CTGCTTAGGG TTAGGCGTTT
301   TGCGCTGCTT CGCGATGTAC GGGCCAGATA TACGCGTTGA CATTGATTAT TGACTAGTTA
361   TTAATAGTAA TCAATTACGG GGTCATTAGT TCATAGCCCA TATATGGAGT TCCGCGTTAC
421   ATAACTTACG GTAAATGGCC CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC
481   AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC GTCAATGGGT
541   GGACTATTTA CGGTAAACTG CCCACTTGGC AGTACATCAA GTGTATCATA TGCCAAGTAC
601   GCCCCCTATT GACGTCAATG ACGGTAAATG GCCCGCCTGG CATTATGCCC AGTACATGAC
661   CTTATGGGAC TTTCCTACTT GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT
721   GATGCGGTTT TGGCAGTACA TCAATGGGCG TGGATAGCGG TTTGACTCAC GGGGATTTCC
781   AAGTCTCCAC CCCATTGACG TCAATGGGAG TTTGTTTTGG CACCAAAATC AACGGGACTT
841   TCCAAAATGT CGTAACAACT CCGCCCCATT GACGCAAATG GGCGGTAGGC GTGTACGGTG
901   GGAGGTCTAT ATAAGCAGAG CTCTCTGGCT AACTAGAGAA CCCACTGCTT ACTGGCTTAT
961   CGAAATTAAT ACGACTCACT ATAGGGAGAC CCAAGCTTGG TACCATGGAA GCCCCAGCTC
1021  AGCTTCTCTT CCTCCTGCTA CTCTGGCTCC CAGATACCAC CGGAGACATT GTTCTGACTC
1081  AGTCTCCAGC CACCCTGTCT GTGACTCCAG GAGATAGAGT CTCTCTTTCC TGCAGGGCCA
1141  GCCAGAGTAT TAGCGACTAC TTACACTGGT ATCAACAAAA ATCACATGAG TCTCCAAGGC
1201  TTCTCATCAA ATATGCTTCC CATTCCATCT CTGGGATCCC CTCCAGGTTC AGTGGCAGTG
1261  GATCAGGGTC AGATTTCACT CTCAGTATCA ACAGTGTGGA ACCTGAAGAT GTTGGAATTT
1321  ATTACTGTCA ACATGGTCAC AGCTTTCCGT GGACGTTCGG TGGAGGCACC AAGCTGGAAA
1381  TCAAACGTAA GTCTCGAGTC TCTAGATAAC CGGTCAATCG GTCAATCGAT TGGAATTCTA
1441  AACTCTGAGG GGGTCGGATG ACGTGGCCAT TCTTTGCCTA AAGCATTGAG TTTACTGCAA
1501  GGTCAGAAAA GCATGCAAAG CCCTCAGAAT GGCTGCAAAG AGCTCCAACA AAACAATTTA
1561  GAACTTTATT AAGGAATAGG GGGAAGCTAG GAAGAAACTC AAAACATCAA GATTTTAAAT
1621  ACGCTTCTTG GTCTCCTTGC TATAATTATC TGGGATAAGC ATGCTGTTTT CTGTCTGTCC
1681  CTAACATGCC CTTATCCGCA AACAACACAC CCAAGGGCAG AACTTTGTTA CTTAAACACC
1741  ATCCTGTTTG CTTCTTTCCT CAGGAACTGT GGCTGCACCA TCTGTCTTCA TCTTCCCGCC
1801  ATCTGATGAG CAGTTGAAAT CTGGAACTGC CTCTGTTGTG TGCCTGCTGA ATAACTTCTA
1861  TCCCAGAGAG GCCAAAGTAC AGTGGAAGGT GGATAACGCC CTCCAATCGG GTAACTCCCA
1921  GGAGAGTGTC ACAGAGCAGG ACAGCAAGGA CAGCACCTAC AGCCTCAGCA GCACCCTGAC
1981  GCTGAGCAAA GCAGACTACG AGAAACACAA AGTCTACGCC TGCGAAGTCA CCCATCAGGG
2041  CCTGAGCTCG CCCGTCACAA AGAGCTTCAA CAGGGGAGAG TGTTAGAGGG AGAAGTGCCC
2101  CCACCTGCTC CTCAGTTCCA GCCTGACCCC CTCCCATCCT TTGGCCTCTG ACCCTTTTTC
2161  CACAGGGGAC CTACCCCTAT TGCGGTCCTC CAGCTCATCT TTCACCTCAC CCCCCTCCTC
2221  CTCCTTGGCT TTAATTATGC TAATGTTGGA GGAGAATGAA TAAATAAAGT GAATCTTTGC
2281  ACCTGTGGTT TCTCTCTTTC CTCATTTAAT AATTATTATC TGTTGTTTTA CCAACTACTC
2341  AATTTCTCTT ATAAGGGACT AAATATGTAG TCATCCTAAG GCACGTAACC ATTTATAAAA
2401  ATCATCCTTC ATTCTATTTT ACCCTATCAT CCTCTGCAAG ACAGTCCTCC CTCAAACCCA
2461  CAAGCCTTCT GTCCTCACAG TCCCCTGGGC CATGGTAGGA GAGACTTGCT TCCTTGTTTT
2521  CCCCTCCTCA GCAAGCCCTC ATAGTCCTTT TTAAGGGTGA CAGGTCTTAC AGTCATATAT
2581  CCTTTGATTC AATTCCCTGA GAATCAACCA AAGCAAATTT TTCAAAAGAA GAAACCTGCT
2641  ATAAAGAGAA TCATTCATTG CAACATGATA TAAAATAACA ACACAATAAA AGCAATTAAA
2701  TAAACAAACA ATAGGGAAAT GTTTAAGTTC ATCATGGTAC TTAGACTTAA TGGAATGTCA
2761  TGCCTTATTT ACATTTTTAA ACAGGTACTG AGGGACTCCT GTCTGCCAAG GGCCGTATTG
2821  AGTACTTTCC ACAACCTAAT TTAATCCACA CTATACTGTG AGATTAAAAA CATTCATTAA
2881  AATGTTGCAA AGGTTCTATA AAGCTGAGAG ACAAATATAT TCTATAACTC AGCAATCCCA
2941  CTTCTAGATG ACTGAGTGTC CCCACCCACC AAAAAACTAT GCAAGAATGT TCAAAGCAGC
3001  TTTATTTACA AAAGCCAAAA ATTGGAAATA GCCCGATTGT CCAACAATAG AATGAGTTAT
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FIG. 14A

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3061 TAAACTGTGG TATGTTTATA CATTAGAATA CCCAATGAGG AGAATTAACA AGCTACAACT
3121 ATACCTACTC ACACAGATGA ATCTCATAAA AATAATGTTA CATAAGAGAA ACTCAATGCA
3181 AAAGATATGT TCTGTATGTT TTCATCCATA TAAAGTTCAA AACCAGGTAA AAATAAAGTT
3241 AGAAATTTGG ATGGAAATTA CTCTTAGCTG GGGGTGGGCG AGTTAGTGCC TGGGAGAAGA
3301 CAAGAAGGGG CTTCTGGGGT CTTGGTAATG TTCTGTTCCT CGTGTGGGGT TGTGCAGTTA
3361 TGATCTGTGC ACTGTTCTGT ATACACATTA TGCTTCAAAA TAACTTCACA TAAAGAACAT
3421 CTTATACCCA GTTAATAGAT AGAAGAGGAA TAAGTAATAG GTCAAGACCA ACGCAGCTGG
3481 TAAGTGGGGG CCTGGGATCA AATAGCTACC TGCCTAATCC TGCCCWCTTG AGCCCTGAAT
3541 GAGTCTGCCT TCCAGGGCTC AAGGTGCTCA ACAAAACAAC AGGCCTGCTA TTTTCCTGGC
3601 ATCTGTGCCC TGTTTGGCTA GCTAGGAGCA CACATACATA GAAATTAAAT GAAACAGACC
3661 TTCAGCAAGG GGACAGAGGA CAGAATTAAC CTTGCCCAGA CACTGGAAAC CCATGTATGA
3721 ACACTCACAT GTTTGGGAAG GGGGAAGGGC ACATGTAAAT GAGGACTCTT CCTCATTCTA
3781 TGGGGCACTC TGGCCCTGCC CCTCTCAGCT ACTCATCCAT CCAACACACC TTTCTAAGTA
3841 CCTCTCTCTG CCTACACTCT GAAGGGGTTC AGGAGTAACT AACACAGCAT CCCTTCCCTC
3901 AAATGACTGA CAATCCCTTT GTCCTGCTTT GTTTTTCTTT CCAGTCAGTA CTGGGAAAGT
3961 GGGGAAGGAC AGTCATGGAG AAACTACATA AGGAAGCACC TTGCCCTTCT GCCTCTTGAG
4021 AATGTTGATG AGTATCAAAT CTTTCAAACT TTGGAGGTTT GAGTAGGGGT GAGACTCAGT
4081 AATGTCCCTT CCAATGACAT GAACTTGCTC ACTCATCCCT GGGGGCCAAA TTGAACAATC
4141 AAAGGCAGGC ATAATCCAGT TATGAATTCT TGCGGCCGCT TGCTAGCTTC ACGTGTTGGA
4201 TCCAACCGCG GAAGGGCCCT ATTCTATAGT GTCACCTAAA TGCTAGAGCT CGCTGATCAG
4261 CCTCGACTGT GCCTTCTAGT TGCCAGCCAT CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT
4321 TGACCCTGGA AGGTGCCACT CCCACTGTCC TTTCCTAATA AAATGAGGAA ATTGCATCGC
4381 ATTGTCTGAG TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG
4441 AGGATTGGGA AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG GCTTCTGAGG
4501 CGGAAAGAAC CAGCTGGGGC TCTAGGGGGT ATCCCCACGC GCCCTGTAGC GGCGCATTAA
4561 GCGCGGCGGG TGTGGTGGTT ACGCGCAGCG TGACCGCTAC ACTTGCCAGC GCCCTAGCGC
4621 CCGCTCCTTT CGCTTTCTTC CCTTCCTTTC TCGCCACGTT CGCCGGGCCT CTCAAAAAAG
4681 GGAAAAAAAG CATGCATCTC AATTAGTCAG CAACCATAGT CCCGCCCCTA ACTCCGCCCA
4741 TCCCGCCCCT AACTCCGCCC AGTTCCGCCC ATTCTCCGCC CCATGGCTGA CTAATTTTTT
4801 TTATTTATGC AGAGGCCGAG GCCGCCTCGG CCTCTGAGCT ATTCCAGAAG TAGTGAGGAG
4861 GCTTTTTTGG AGGCCTAGGC TTTTGCAAAA AGCTTGGACA GCTCAGGGCT GCGATTTCGC
4921 GCCAAACTTG ACGGCAATCC TAGCGTGAAG GCTGGTAGGA TTTTATCCCC GCTGCCATCA
4981 TGGTTCGACC ATTGAACTGC ATCGTCGCCG TGTCCCAAAA TATGGGGATT GGCAAGAACG
5041 GAGACCTACC CTGGCCTCCG CTCAGGAACG AGTTCAAGTA CTTCCAAAGA ATGACCACAA
5101 CCTCTTCAGT GGAAGGTAAA CAGAATCTGG TGATTATGGG TAGGAAAACC TGGTTCTCCA
5161 TTCCTGAGAA GAATCGACCT TTAAAGGACA GAATTAATAT AGTTCTCAGT AGAGAACTCA
5221 AAGAACCACC ACGAGGAGCT CATTTTCTTG CCAAAAGTTT GGATGATGCC TTAAGACTTA
5281 TTGAACAACC GGAATTGGCA AGTAAAGTAG ACATGGTTTG GATAGTCGGA GGCAGTTCTG
5341 TTTACCAGGA AGCCATGAAT CAACCAGGCC ACCTTAGACT CTTTGTGACA AGGATCATGC
5401 AGGAATTTGA AAGTGACACG TTTTTCCCAG AAATTGATTT GGGGAAATAT AAACTTCTCC
5461 CAGAATACCC AGGCGTCCTC TCTGAGGTCC AGGAGGAAAA AGGCATCAAG TATAAGTTTG
5521 AAGTCTACGA GAAGAAAGAC TAACAGGAAG ATGCTTTCAA GTTCTCTGCT CCCCTCCTAA
5581 AGCTATGCAT TTTTATAAGA CCATGGGACT TTTGCTGGCT TTAGATCTCT TTGTGAAGGA
5641 ACCTTACTTC TGTGGTGTGA CATAATTGGA CAAACTACCT ACAGAGATTT AAAGCTCTAA
5701 GGTAAATATA AAATTTTTAA GTGTATAATG TGTTAAACTA CTGATTCTAA TTGTTTGTGT
5761 ATTTTAGATT CCAACCTATG GAACTGATGA ATGGGAGCAG TGGTGGAATG CCTTTAATGA
5821 GGAAAACCTG TTTTGCTCAG AAGAAATGCC ATCTAGTGAT GATGAGGCTA CTGCTGACTC
5881 TCAACATTCT ACTCCTCCAA AAAAGAAGAG AAAGGTAGAA GACCCCAAGG ACTTTCCTTC
5941 AGAATTGCTA AGTTTTTTGA GTCATGCTGT GTTTAGTAAT AGAACTCTTG CTTGCTTTGC
6001 TATTTACACC ACAAAGGAAA AAGCTGCACT GCTATACAAG AAAATTATGG AAAAATATTC
6061 TGTAACCTTT ATAAGTAGGC ATAACAGTTA TAATCATAAC ATACTGTTTT TTCTTACTCC
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FIG. 14B

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6121 ACACAGGCAT AGAGTGTCTG CTATTAATAA CTATGCTCAA AAATTGTGTA CCTTTAGCTT
6181 TTTAATTTGT AAAGGGGTTA ATAAGGAATA TTTGATGTAT AGTGCCTTGA CTAGAGATCA
6241 TAATCAGCCA TACCACATTT GTAGAGGTTT TACTTGCTTT AAAAAACCTC CCACACCTCC
6301 CCCTGAACCT GAAACATAAA ATGAATGCAA TTGTTGTTGT TAACTTGTTT ATTGCAGCTT
6361 ATAATGGTTA CAAATAAAGC AATAGCATCA CAAATTTCAC AAATAAAGCA TTTTTTTCAC
6421 TGCATTCTAG TTGTGGTTTG TCCAAACTCA TCAATGTATC TTATCATGTC TGGATCGGCT
6481 GGATGATCCT CCAGCGCGGG GATCTCATGC TGGAGTTCTT CGCCCACCCC AACTTGTTTA
6541 TTGCAGCTTA TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTTCACA AATAAAGCAT
6601 TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT CAATGTATCT TATCATGTCT
6661 GTATACCGTC GACCTCTAGC TAGAGCTTGG CGTAATCATG GTCATAGCTG TTTCCTGTGT
6721 GAAATTGTTA TCCGCTCACA ATTCCACACA ACATACGAGC CGGAAGCATA AAGTGTAAAG
6781 CCTGGGGTGC CTAATGAGTG AGCTAACTCA CATTAATTGC GTTGCGCTCA CTGCCCGCTT
6841 TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT CGGCCAACGC GCGGGGAGAG
6901 GCGGTTTGCG TATTGGGCGC TCTTCCGCTT CCTCGCTCAC TGACTCGCTG CGCTCGGTCG
6961 TTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA TCCACAGAAT
7021 CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC AGGAACCGTA
7081 AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG CATCACAAAA
7141 ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC CAGGCGTTTC
7201 CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC GGATACCTGT
7261 CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG CTCACGCTGT AGGTATCTCA
7321 GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC GTTCAGCCCG
7381 ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA CACGACTTAT
7441 CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA GGCGGTGCTA
7501 CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA TTTGGTATCT
7561 GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA TCCGGCAAAC
7621 AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG CGCAGAAAAA
7681 AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG TGGAACGAAA
7741 ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC TAGATCCTTT
7801 TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT TGGTCTGACA
7861 GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT CGTTCATCCA
7921 TAGTTGCCTG ACTCCCCGTC GTGTAGATAA CTACGATACG GGAGGGCTTA CCATCTGGCC
7981 CCAGTGCTGC AATGATACCG CGAGACCCAC GCTCACCGGC TCCAGATTTA TCAGCAATAA
8041 ACCAGCCAGC CGGAAGGGCC GAGCGCAGAA GTGGTCCTGC AACTTTATCC GCCTCCATCC
8101 AGTCTATTAA TTGTTGCCGG GAAGCTAGAG TAAGTAGTTC GCCAGTTAAT AGTTTGCGCA
8161 ACGTTGTTGC CATTGCTACA GGCATCGTGG TGTCACGCTC GTCGTTTGGT ATGGCTTCAT
8221 TCAGCTCCGG TTCCCAACGA TCAAGGCGAG TTACATGATC CCCCATGTTG TGCAAAAAAG
8281 CGGTTAGCTC CTTCGGTCCT CCGATCGTTG TCAGAAGTAA GTTGGCCGCA GTGTTATCAC
8341 TCATGGTTAT GGCAGCACTG CATAATTCTC TTACTGTCAT GCCATCCGTA AGATGCTTTT
8401 CTGTGACTGG TGAGTACTCA ACCAAGTCAT TCTGAGAATA GTGTATGCGG CGACCGAGTT
8461 GCTCTTGCCC GGCGTCAATA CGGGATAATA CCGCGCCACA TAGCAGAACT TTAAAAGTGC
8521 TCATCATTGG AAAACGTTCT TCGGGGCGAA AACTCTCAAG GATCTTACCG CTGTTGAGAT
8581 CCAGTTCGAT GTAACCCACT CGTGCACCCA ACTGATCTTC AGCATCTTTT ACTTTCACCA
8641 GCGTTTCTGG GTGAGCAAAA ACAGGAAGGC AAAATGCCGC AAAAAAGGGA ATAAGGGCGA
8701 CACGGAAATG TTGAATACTC ATACTCTTCC TTTTTCAATA TTATTGAAGC ATTTATCAGG
8761 GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA CAAATAGGGG
8821 TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTC
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FIG. 14C

VK Domain

CD40 DIVLTQSPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRLLIK

VKIII EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLLIY

CD40 YASHSISGIPSRFSGSGSGSDFTLSINSVEPEDVGIYYCQHGHSFPWTFGGGTKLEIK

VKIII/JK4 DASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWPLTFGGGTKVEIK

VH Domain

CD40 QIQLVQSGPELKKPGETVRISCKASGYAFTTTGMQWVQEMPGKGLKWIG

VH7 QVQLVQSGSELKKPGASVKVSCKASGYTFTSYAMNWVRQAPGQGLEWMG

CD40 WINTHSGVPKYVEDFKGRFAFSLETSANTAYLQISNLKNEDTATYFCVR

VH7 WINTNTGNPTYAQGFTGRFVFSLDTSVSTAYLQISSLKAEDTAVYYCAR

CD40 SGNGNYDLAYFAYWGQGTLVTVSA

JH4 YFDYWGQGTLVTVSS

FIG. 15

FIG. 16

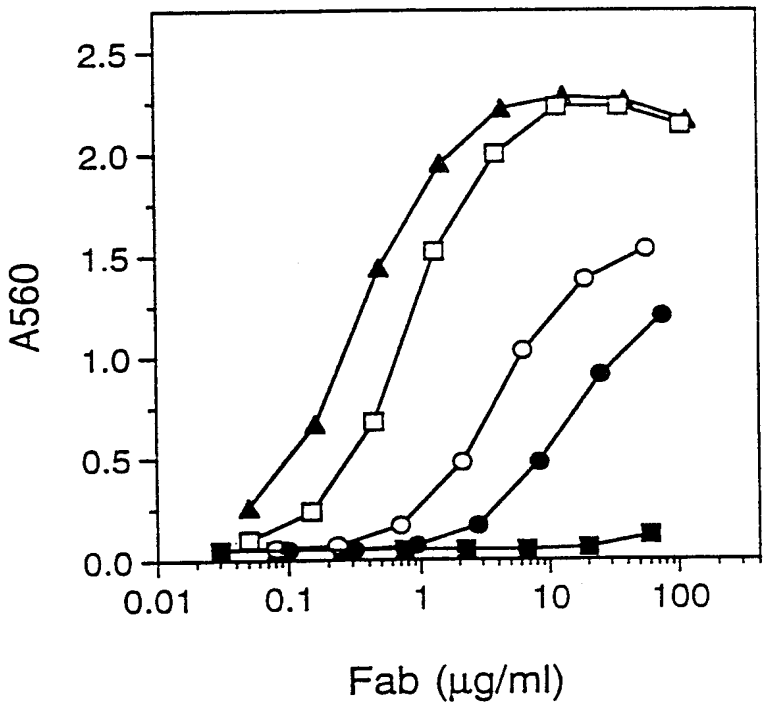

FIG. 17

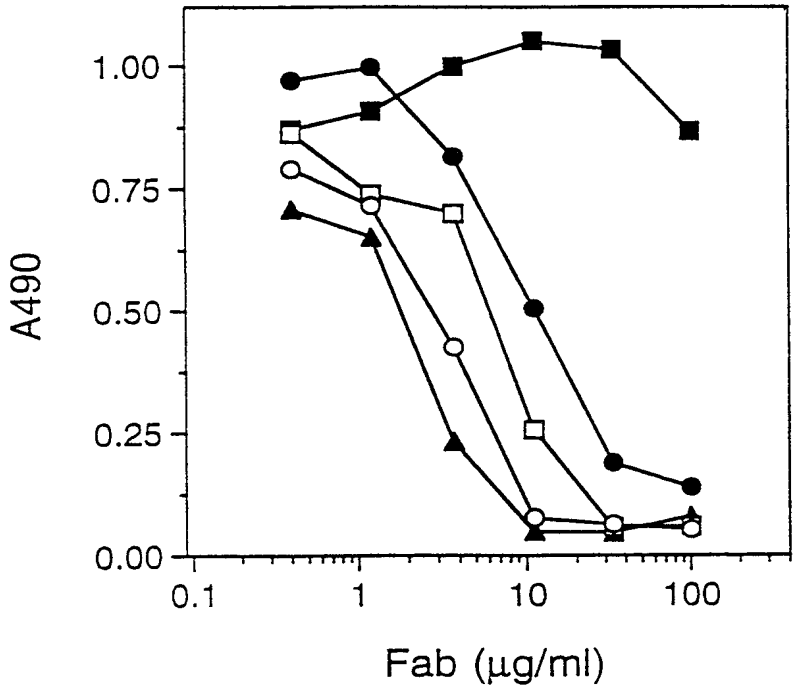

FIG. 18A

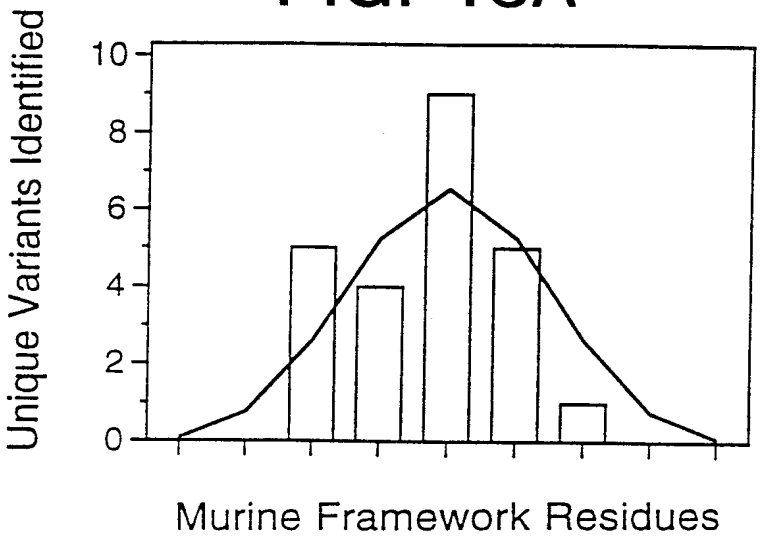

FIG. 18B